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mRNA BINDING FACTOR MEDIATED mRNA:RIBOSOME INITIATION COMPLEX FORMATION IN THE RABBIT LIVER CELL-FREE PROTEIN SYNTHESIZING SYSTEM

by

© MICHAEL J.P. DUFRESNE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "mRNA Binding Factor Mediated mRNA:Ribosome Initiation Complex Formation in the Rabbit Liver Cell-Free Protein Synthesizing System", submitted by Michael J.P. Dufresne in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

Supervisor

External Examiner

Date October 10, 1974.

to my parents

ABSTRACT

The mechanism of messenger RNA binding to ribosomes was investigated in a rabbit liver cell-free system in which all reaction components were purified to homogeneity and free of RNase. The biological significance of the observed mRNA:ribosome complex was examined in terms of specific binding of aminoacyl-tRNA to the mRNA:ribosome complex, as specified by the genetic codon of the bound mRNA.

In order to study mRNA:ribosome complex formation and specific binding of aminoacyl-tRNA, at least four reaction components were necessary: tRNA, aminoacyl-tRNA synthetase, mRNA, and ribosomes. The first two components were required to prepare aminoacyl-tRNA; the last two components were required to form a mRNA:ribosome complex. When these studies were initiated in 1970, there were no established methods for the isolation of the reaction components. Therefore it was necessary to prepare rabbit liver - tRNA (Chapter 3), - aminoacyl-tRNA synthetase (Chapter 4), and - ribosomes (Chapter 5) to homogeneity. The preparation of mRNA was avoided by using synthetic homopolyribonucleates.

The ribosomes prepared were found to be incapable of binding any mRNA's. The capacity to bind mRNA was found to be separated from the ribosomes during the purification procedure. This fact led to the discovery of protein factors which mediate the binding of synthetic mRNA's to the ribosomes (Chapter 6).

The mRNA binding proteins can be separated into four functional groups, each of which mediates the binding of one of poly-A, poly-C, poly-G, or poly-U respectively. Of these four proteins, the poly-A specific binding factor (M_A-factor) and the poly-U specific binding factor (M_U-factor) were purified to homogeneity. The molecular weight of both factors was determined as 60,000 daltons using SDS-gel electrophoresis. The M-factor mediated binding of homopolynucleates is not an artifact since the ternary complex - mRNA:ribosome:M-factor, binds with aminoacyl-tRNA as specified by the genetic code. However, the binding of aminoacyl-tRNA to the ternary complex requires an additional factor.

The mechanism of ATA inhibition on the formation of the ternary complex was also examined. ATA, a known inhibitor of mRNA:ribosome interaction in other systems, inhibits the formation of the mRNA: M-factor complex, but does not inhibit the binding of this complex to ribosomes.

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LIST OF ABBREVIATIONS

A	adenosine
C	cytidine
G	guanosine
U	uridine
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CMP	cytidine 5'-monophosphate
CTP	cytidine 5'-triphosphate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
poly-A	poly-adenosine monophosphate
poly-C	poly-cytidine monophosphate
poly-G	poly-guanosine monophosphate
poly-U	poly-uridine monophosphate
poly-AU	mixed polymer of adenosine and uridine
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein complex
tRNA	transfer ribonucleic acid
$tRNA_f^{met}$	transfer ribonucleic acid specific for a methionine which can be formylated
$tRNA_m^{met}$	transfer ribonucleic acid specific for a methionine which cannot be formylated
aa-tRNA	aminoacyl-transfer ribonucleic acid

fmet-tRNA$_f^{met}$	formyl-methionyl-transfer ribonucleic acid
lys-tRNA	lysyl-transfer ribonucleic acid
phe-tRNA	phenylalanyl-transfer ribonucleic acid
DNase I	bovine pancreatic deoxyribonuclease I
RNase A	bovine pancreatic ribonuclease A
GTPase	guanosine triphosphatase
CoA	coenzyme A
ATA	aurintricarboxylic acid
βME	β-mercaptoethanol
DOC	deoxycholate
EDTA	ethylenediaminetetraacetate
PEP	phosphoenol pyruvate
SDS	sodium dodecyl sulfate
TCA	trichloracetic acid
Tris	tris (hydroxymethyl) amino methane
DEAE-	diethylaminoethyl-
-SH	sulfhydryl group
PP_i	inorganic pyrophosphate
cpm	counts per minute
rpm	revolutions per minute
ds	double-stranded
ss	single-stranded
UV	ultraviolet
A_n	absorbance at n nm
MW	molecular weight

M	Molar
µmoles	micromoles
nmoles	nanomoles
µµ moles	micro-micromoles
pmoles	picomoles
g	grams
mg	milligrams
µg	micrograms
ng	nanograms
ml	milliliters
µl	microliters
mm	millimeters
cm	centimeters
nm	nanometers
sec	seconds
min	minutes
hr	hours
°C	degrees centigrade
S	Svedberg
$s_{20,w}$	sedimentation coefficient corrected to 20°C for solvent viscosity and density
I_{50}	concentration of inhibitor sufficient to cause 50% inhibition of a reaction

CHAPTER 1

INTRODUCTION

The discovery of tRNA in 1955 by Zamecnik and co-workers (1,2) and the establishment of the cell-free hemoglobin synthesizing system in 1958 by Schweet and co-workers (3), signalled the introduction of studies of the *in vitro* translation of genetic information. These studies, developed from eukaryotic cells, provided one of the most significant means of investigating the mechanism of protein synthesis. Because of the difficulty in obtaining cell-free preparations from bacteria, the early studies of protein synthesis were carried out almost exclusively in mammalian systems. It was not until 1960 that Lamborg and Zamecnik reported the successful use of an *E. coli* cell-free system for examining protein synthesis (4).

Once the cell-free *E. coli* system was established, a new wave of investigations began and the rate of discovery of new facts took an abrupt turn upwards. Concepts originally conceived in mammalian systems were quickly applied and followed up in the bacterial system. As a result, a difference in progress between the two systems emerged during the 1960's. By the end of the decade it was obvious that with respect to research into the mechanism of protein synthesis, advances made in the mammalian system were small compared to those made in the bacterial system.

I. Progress in Bacterial Protein Synthesis (1960-1970)

The following concepts were well established in the bacterial system when this thesis work was initiated in 1970:

(i) It was established that two species of met-tRNA^{met} exist in E. coli. One species, met-tRNA$_f^{met}$, can be formylated whereas the other, met-tRNA$_m^{met}$, cannot (5,6). These two species, although synthesized by the same methionyl-tRNA synthetase (7,8), display different functions: tRNA$_f^{met}$ delivers a formylated methionine into the N-terminal position of proteins (9,10), whereas tRNA$_m^{met}$ delivers an unformylated methionine into internal positions of the growing polypeptide chain (11). Subsequent studies showed that fmet-tRNA$_f^{met}$ functions as the chain initiator amino-acyl-tRNA for E. coli polypeptide synthesis (12-14), and recognizes the initiation codons AUG and GUG (5,11).

(ii) The reaction mechanism of aminoacyl-tRNA synthesis was well studied. The reaction is catalyzed by aminoacyl-tRNA synthetases specific for a particular amino acid and requires the presence of ATP and Mg^{2+}. By 1970, many aminoacyl-tRNA synthetases had been isolated from bacterial systems (15-21).

(iii) The behavior of ribosomal subunits in bacterial peptide synthesis was also established. Experiments using differential labelling of ribosomal populations with radioisotopes (^3H, ^{14}C), demonstrated that essentially all the ribosomal subunits exchange with polysomes (a number of ribosomes attached to the same mRNA) (22-25). Additional isotope studies provided firm evidence for the obligatory formation of an

initiation complex involving the specific binding of mRNA and fmet-$tRNA_f^{met}$ to the 30S ribosomal subunit (26,27).

(iv) Protein factors necessary for the translation of natural and synthetic mRNA's were isolated and characterized in several laboratories (28-31). Despite the confusion caused by the various designations assigned to these factors, there are essentially three protein factors involved in the complex formation during the initial step of protein synthesis. These factors are localized predominantly on the 30S ribosomal subunit (32,33). Table 1 summarizes the properties and functions of the three initiation factors.

(v) Stepwise addition of amino acids to a nascent peptide chain is the essence of polypeptide elongation. The elongation process consists of three major events: the first, factor mediated binding of aminoacyl-tRNA to the ribosome:mRNA complex; the second, addition of the amino acid to the carboxyl terminal of the growing polypeptide chain; the third, concomitant translocation of mRNA and newly synthesized peptidyl-tRNA (34-36). There are three factors involved in the elongation process: Elongation Factor (EF)-T_u, -T_s, and -G. Additional proteins displaying peptide bond forming activity are reported to be associated with the large, 50S, ribosomal subunit (37). Table 1 summarizes the properties and functions of these elongation factors.

(vi) Termination of polypeptide synthesis was also studied during the 1960's. Several lines of evidence demonstrate the existence of specific termination codons. Studies in E. coli involving various

TABLE I

Properties and Function of Factors Involved in Protein Synthesis 1960-70

BACTERIAL					MAMMALIAN				
Factor		Physical Properties	Function	Ref.	Factor	Source	Physical Properties	Function	Ref.
INITIATION									
IF-1	(F1,A, FI)*	9,000	acts with IF-2 in AUG dependent binding of $fMET\text{-}tRNA_f^{met}$ to ribosomes	(47,52, 54)	M_1,M_2	rabbit ret.		lowers optimum magnesium conc. for polyphenyl-alanine synthesis in rabbit ret.	111,113-115
IF-2	(F2,C, FIII)	60,000-80,000 SH-groups essential for activity	stimulates binding of ribosomes to mRNA; attachment of fMET-tRNA to the 30S:mRNA complex (GTP dependent)**	(47,49-51,53, 55-61)					
IF-3	(F3,B, FII)	29,000	involved in formation of initiation complex when natural mRNA is template	(31,49, 50,54, 62)	M_3			for ret. system appears to be required for natural mRNA translation	115
ELONGATION									
T***									
T_u****	(S,FI_u, TI_u)	~40,000 binds GDP strongly stabilized by Mg^{2+} 3 SH-groups	formation of a T_u:GTP: aa-tRNA complex intermediate in the binding of aa-tRNA's to the ribosomes	(34,63-76)	T1	rabbit ret.	186,000 (rabbit ret.) 3 subunits each MW 62,000	binding of aa-tRNA to ribosomes - GTPase activity	116-121

BACTERIAL					MAMMALIAN				
Factor		Physical Properties	Function	Ref.	Factor	Source	Physical Properties	Function	Ref.
ELONGATION (con't)									
T_s	(S_1,FI_s TI_s)	~20,000	displaces GDP from T_u:GDP complex. Permits formation of T_u:T_s complex which is able to interact with aa-tRNA and GTP to form T_u:GTP:aa-tRNA	(63,64, 66-68, 77)					
G	(S_2,FII TII)	70-85,000 activity depends on free thiol groups	mediates the GTP dependent translocation of the peptidyl-tRNA to the donor site thereby leaving the acceptor site free to accept another aa-tRNA	(63,64, 78-93)	T2	rat liver rabbit ret.	60-65,000 (rat liver)	complements T1 in polymerization ribosome dependent GTPase activity GTP dependent translocation of the peptidyl-tRNA (see G factor)	121-123
peptidyl transferase	(peptide synthetase)	catalytic centre on 50S subunit	catalyzes the peptide bond formation between the α-amino group of the aa-tRNA with the carboxyl terminal of neighboring peptidyl tRNA	(37,94, 95)	peptidyl transferase	human tonsils	activity localized on 60S subunit	see E. coli peptidyl transferase	124

BACTERIAL					MAMMALIAN				
Factor		Physical Properties	Function	Ref.	Factor	Source	Physical Properties	Function	Ref.
TERMINATION									
R_1	R_1&R_2 separable by low then high potassium phosphate conc. elution from Ca. phosphate	44,000	necessary for translation of codons UAA and UAG	(42-45)	R	rabbit ret.	stimulates	stimulates release of fMet from fMet-tRNA: ribosome intermediate in presence of a polynucleotide template containing the bases U and A	112
R_2		47,000	required to translate UAA and UGA						
S	α	46,000	No release activity. stimulates rate but not extent of the release dependent on a particular R factor & appropriate termination codon						

* other designations for the same factor

** mRNA binding activity and the fMet-tRNA binding activity of F2 could not be separated. Activities, however, are independent (96)

*** T_u and T_s are associated in the soluble fraction of the cell and called T factor (97)

**** Subscripts u and s refer to "unstable" and "stable" respectively as determined by heat lability.

gene mutations, such as amber and ochre, led to the identification of UAG, UAA, and UGA as termination codons (38-41). Proteins responsible for the termination codon-dependent release of peptidyl-tRNA were also isolated and characterized before 1970 (42-46) (Table 1).

II. Progress in Mammalian Protein Synthesis (1960-1970)

From the above summation of progress for the E. coli system, it is obvious that by 1970 substantial advances had been made concerning the major events of bacterial protein synthesis: initiation, elongation, and termination. In contrast to this, very little progress had been made concerning the mechanism of mammalian protein synthesis as described in the following summation:

(i) The initiation mechanism was not yet determined. Although evidence for the existence of two different $tRNA^{met}$ species was presented (98), there was no conclusive evidence for the presence of an initiator aminoacyl-tRNA similar to bacterial fmet-$tRNA_f^{met}$. Studies in the rabbit reticulocyte cell-free system (99), and the trout testis system (100), suggested that the N-terminal amino acid of both hemoglobin and protamine synthesized in cell suspensions was methionine. However, no further progress was made concerning these studies.

(ii) Despite the fact that the function of tRNA as a specific amino acid accepting molecule was discovered in the mammalian system (101), the enzyme catalyzing this reaction was not characterized until after 1970. It is interesting to note that at present, only three mammalian aminoacyl-tRNA synthetases have been characterized (102-105).

(iii) The role of ribosomal subunits was not yet known. The participation of the small, 40S, ribosomal subunit in an initiation complex was suggested from studies of rabbit reticulocutes (106) and HeLa cells (107). However, this conclusion is based on the observation that exchange occurs between the pool of ribosomal subunits, ribosomes and polysomes in mammalian systems (106, 108-110). The cause for this delayed progress was largely due to the difficulty in dissociating mammalian ribosomes. Lowering the Mg^{2+} concentration, which dissociates bacterial ribosomes into biologically active subunits, is not effective in mammalian ribosomes systems.

(iv) The participation of protein factors in protein synthesis was originally discovered for the reticulocyte system. However, progress in the mammalian system was delayed due to the inability to prepare ribosomal subunits necessary for assigning the functions of individual protein factors. With respect to initiation factors, a fraction termed M was obtained from crude rabbit reticulocyte ribosomes by extraction with high salt (111). This crude fraction was found to stimulate the initiation of globin synthesis. Purification of this fraction into distinct initiation activities comparable to E. coli IF-1, -2, and -3 occurred only after 1970. Table 1 summarizes the properties of M fraction.

(v) A notable contribution from the mammalian system during the 1960's was the establishment of the direction of polypeptide elongation from

the N-terminal to the carboxyl-terminal of the polypeptide chain. Considerable progress was also made in the characterization of protein factors involved in the elongation process. Before 1970, two elongation factors had been partially purified and characterized. One of these factors corresponds to E. coli T factor and the other to the E. coli G factor. The properties of these factors are summarized in Table 1.

(vi) During the 1960's little was reported concerning the termination mechanism in mammalian protein synthesis. Evidence for termination codon(s) is still not available; however, a termination-associated release factor has been reported (112).

III. Project Formulation

It is apparent that for all aspects of protein synthesis considered, progress in the mammalian system lagged behind progress in the E. coli system.

Why did this difference in progress exist between the two systems? One reason was the difficulty in dissociating ribosomes into subunits, which is necessary for examining the mechanism of protein synthesis. Another reason was the difficulty in developing techniques and methodology for purification and maintenance of other components in a biologically active form.

In 1970, we initiated studies on protein synthesis in a rabbit liver cell-free system. These studies were undertaken to gain a more comprehensive picture of mammalian protein synthesis and to permit a detailed comparison of this system with the bacterial system. Our main

interest was the initial steps of protein synthesis; in particular, determination of the mechanism and the biological significance of *in vitro* mRNA:ribosome complex formation. By focusing our attention on the mRNA:ribosome complex we eliminated many of the problems inherent in examining a complete protein synthesizing system. Figure 1 illustrates in simple form the synthetic mRNA:ribosome initiation complex.

To analyze the steps leading to the mRNA:ribosome complex we decided to establish a completely homologous cell-free system free of RNase and consisting of components purified from rabbit liver. The reason for these requirements was based on our observation that the use of impure and/or heterologous components decreased the reliability in assigning the function of individual components in the initiation complex formation.

Analysis of the mechanism of mRNA:ribosome complex formation required two basic approaches: (1) the demonstration of mRNA:ribosome complex formation under appropriate conditions, and (2) confirmation of the biological significance of the mRNA:ribosome complex. Since the specific binding of aminoacyl-tRNA to the mRNA:ribosome complex is the next event during protein synthesis, the significance of the interaction between mRNA and ribosomes was assessed by the binding of aminoacyl-tRNA to the complex as specified by the genetic codons on the bound mRNA.

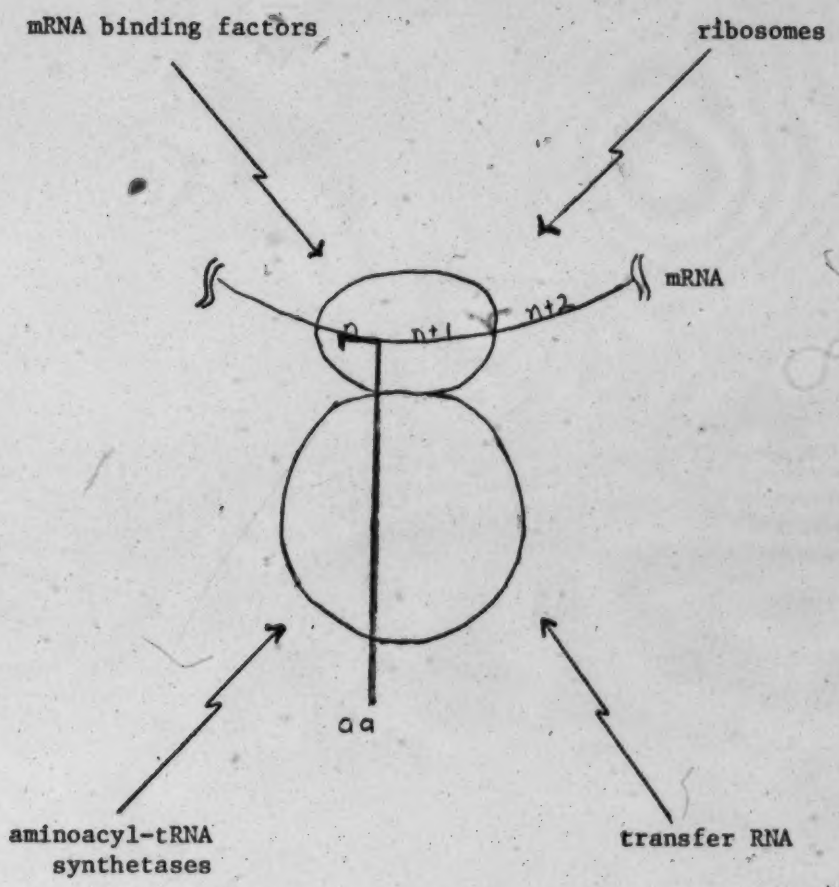


FIGURE 1: Messenger RNA:Ribosome complex indicating a bound aminoacyl-tRNA (n, n+1, and n+2 designate genetic codons on the bound mRNA).

IV. Establishment of an Homologous mRNA-Ribosome system

In pursuing the above two lines of experiments at least four (possibly five) reaction components were required: tRNA, aminoacyl-tRNA synthetase, mRNA, ribosomes and, if necessary, protein factors. The first two components were required for the preparation of amino-acyl-tRNA for the specific binding experiments. The mRNA and purified ribosomes were necessary to demonstrate mRNA:ribosome complex formation. For this latter study we chose a series of commercial homopolyribo-nucleates as mRNA; however, the ribosomes had to be purified from rabbit liver. The requirement for specific protein factors to mediate mRNA:ribosome complex formation was not known when we initiated our experiments; nevertheless, in light of progress in the E. coli system we anticipated the necessity of some protein factor(s) in our system.

The preparation of gram quantities of rabbit liver tRNA was accomplished and is described in Chapter 3 (125). Since the preparation of large quantities of liver tRNA was elaborative, it was used only in the final stages of each experiment. During the preliminary stage of the experiments, commercial yeast tRNA was employed as a substitute. In this regard, it should be mentioned that yeast tRNA is not as effective as homologous rabbit liver tRNA in the aminoacylation reaction.

The necessity for obtaining purified aminoacyl-tRNA synthetase was recognized at the early stages of our research. In the presence of crude protein from a post-ribosomal fraction neither liver tRNA nor yeast tRNA was able to accept sufficient amino acid to permit isolation of labelled aminoacyl-tRNA. We therefore attempted an extensive

purification of two synthetases: phenylalanyl-tRNA synthetase and lysyl-tRNA synthetase. Both of these synthetases were purified. Phenylalanyl-tRNA synthetase was purified to homogeneity and studied in depth as described in Chapter 4 (126). Using these purified enzymes we could obtain sufficient quantities of phe-tRNA and lys-tRNA for use in studies of specific binding of aminoacyl-tRNA to the mRNA:ribosome complex.

Due to the instability of the mammalian ribosome structure, the preparation of pure rabbit liver ribosomes required considerable modification of the methods previously employed for the isolation of bacterial ribosomes. A method for the large scale preparation of pure rabbit liver ribosomes was established as described in Chapter 5 (127). This method includes the application of differential centrifugation of tissue homogenate, deoxycholate treatment of a 150,000 x G supernatant, concentration of ribosomes by partition, and purification of ribosomes by DEAE-cellulose chromatography.

The most striking feature of the purified ribosomes was that they were unable to bind any mRNA tested, although they were active in protein synthesis when supplemented with crude protein from a post-ribosomal supernatant. This finding led us to the discovery of messenger RNA binding factors from post-ribosomal supernatant proteins as described briefly in the following section and in detail in Chapter 6 (128). The isolation of mRNA binding factors and examination of their role in mRNA:ribosome complex formation represent the major topics of this thesis.

V. Discovery of mRNA Binding Factors

The lack of mRNA binding capacity of the purified ribosomes suggested to us that the purified ribosomal system required the presence of some

additional component(s) to mediate the mRNA binding. Since the purified ribosomes were active in protein synthesis when supplemented with a crude protein fraction it was obvious that mRNA:ribosome complex formation must have occurred. Moreover, the absolute requirement for the protein fraction suggested to us that this fraction might contain the mRNA binding activity. In keeping with our requirement for a homologous system, we initiated a rigorous search for mRNA binding factor(s) from rabbit liver tissue. The most logical approach was to determine at what stage of purification the ribosomes lost the capacity to bind mRNA. In fact we found poly-U binding factor predominantly in the first post-ribosomal supernatant and some residual activity in the crude ribosomal fraction. The most interesting feature of this poly-U binding factor was that it was able to mediate the binding of only poly-U to the purified ribosomes. No other homopolynucleate binding could be demonstrated in the presence of this protein. This observation at the early stage of our investigation led us to search for a full complement of mRNA binding factors specific for each homopolynucleate.

Four specific mRNA binding factors, one for each of the homopolymers poly-A, -C, -G, and -U were detected in crude fractions. These factors were designated as M_A-, M_C-, M_G-, and M_U- factor, respectively. The stability of these four binding factors varied tremendously depending on the conditions employed for isolation. As a result, we were able to purify only two of the factors to homogeneity, one specific for poly-A and the other specific for poly-U. Unfortunately, during the course of purification of poly-A and poly-U binding factors, poly-C and poly-G factor were inactivated.

The use of poly-A and poly-U binding factors permitted us to demonstrate both poly-A:ribosome and poly-U:ribosome complex formation. As planned at the outset of our program, the biological significance of the mRNA:ribosome:factor complex was verified by the ability of the ternary complex to accept specific aminoacyl-tRNA's: 1. phe-tRNA binding to the poly-U:ribosome M_U-factor complex and, 2. lys-tRNA binding to the poly-A:ribosome M_A-factor complex. These tests demonstrated that binding of aa-tRNA to the ternary complex occurs only when the system is supplemented with GTP and an elongation factor. Thus the significance of the mRNA binding factors, M_A and M_U, for poly-A and poly-U binding, respectively, was confirmed.

VI. Comparison of mRNA Binding Factors, M_A and M_U, with Known Protein Factors

The characterization of the mRNA binding factors, M_A and M_U, provided us with the information necessary to compare and correlate their physical properties and biological function to those of known factors. For the purpose of comparison, Table 2 summarizes the properties and functions of factors described to date in mammalian protein synthesis. For convenience, the mRNA binding factors described in this thesis are also included in the table.

M_A- and M_U-binding factors do not resemble initiation factor IF-M_1, IF-M_2 or elongation factor EF-2 from mammalian sources. However, the binding factors do share some properties of both elongation factor EF-1 and initiation factor IF-M_3 from rabbit reticulocytes. The molecular weight of EF-1 subunits is very close to that obtained for both M_A- and M_U-

TABLE 2

Factors Involved in Mammalian Protein Synthesis (1970-1974)

Factor		Elution from DEAE cellulose	Properties	Source	Function	Ref.
INITIATION						
$IF\text{-}M_1$	(IF-E)*	does not bind	95,000	rabbit ret. ribosomes, liver ribosomes, mouse L-cell		129-132, 230
$IF\text{-}M_2$	($IF\text{-}E_2$)	220 mM-260 mM salt		rabbit ret. ribosome	both required for AUG-dependent binding of met-tRNA to washed ribosomes and for initiation of globin synthesis.	133-135
$IF\text{-}M_{2A}$			excluded on Sephadex G-200 GTPase activity			
$IF\text{-}M_{2B}$			~60,000 (133,134) ~20,000 (129)	liver ribosome		
$IF\text{-}M_3$	($IF\text{-}E_3$)	160-220 mM salt	voided on Sephadex G-200 <25,000	rabbit ret. ribosomes	binding of natural mRNA to ribosomes	135
	F3	comparable to M3		Chick embryo muscle ribosomes	promotes binding of 26S myosin mRNA to 40S subunits	136,137

Factor	Elution from DEAE cellulose	Properties	Source	Function	Ref.
ELONGATION					
EF1	250 mM salt	186,000 3 subunits (α_1) MW 62,000	rabbit ret.	see Table 1	
EF2	100 mM salt	70,000 to 85,000	rabbit & rat liver	translation process (see Table 1)	141,142
			rabbit ret., calf brain, human tonsils		123,143, 144
peptidyl transferase	see Table 1				
TERMINATION	No new information since Table 1				
			guinea pig liver, hamster liver		145
ADDITIONAL FACTORS					
Dissociation factor	-	distinct from IF-M_3	rat liver ribosomal subunits	dissociation of rat liver monomeric ribosomes into 60S and 40S subunits	146
	190 mM salt	distinct from initiation and elongation factors	rabbit ret. ribosomes	promotes dissociation of ribosomes into ribosomal subunits	147,148

Factor	Elution from DEAE cellulose	Properties	Source	Function	Ref
M-FACTORS DESCRIBED IN THIS THESIS					
poly-A binding factor	168 mM salt	60,000 voided on Sephadex G-200 aggregates from Sepharose 6B	rabbit liver	specific binding of polyadenylate to ribosomes	128
poly-U binding factor	220 mM salt	60,000 voided on Sephadex G-200 aggregates from Sepharose 6B	rabbit liver	specific binding of polyuridylate to ribosomes	

* Other designations of the same factor.

according to neutral SDS-polyacrylamide gel electrophoresis. However, EF-1 is included on Sephadex G-200 whereas both M_A- and M_U- factors are excluded. Moreover, EF-1 and the mRNA binding factors differ functionally. M_A- and M_U- factors are completely free of the EF-1 capacity to mediate the binding of aminoacyl-tRNA to the mRNA:ribosome complex.

Of the known factors involved in mammalian protein synthesis, M_A- and M_U- factors most closely resemble initiation factor IF-M_3. In the first place, elution of both IF-M_3 and M-factors occurs between 160 and 220 mM salt. Secondly, both species are excluded from Sephadex G-200; and thirdly, both are involved with the binding of mRNA to the ribosomes. M_A- and M_U-factors, however, show significant differences from IF-M_3. The estimated molecular weight of IF-M_3 is less than 25,000 while that of the mRNA binding factors is 60,000. IF-M_3 does not bind synthetic mRNA to ribosomes; our factors, M_A- and M_U-, do. IF-M_3 is reported to be natural mRNA-specific; binding factors M_A- and M_U- appear to be base-specific.

On the basis of this comparison, it appears that M_A- and M_U- factors differ significantly from other factors known to be involved in mammalian protein synthesis. Although M_A- and M_U- factors differ from other well-characterized proteins as mentioned above, two possibilities concerning functional correlation remain:

(i) M_A- and M_U- factors might represent the mammalian ribosomal protein equivalent to *E. coli* 30S ribosomal protein S1. This protein is acidic in nature, has a molecular weight of 60,000 - 65,000 and is known to comprise the active site for mRNA binding on the 30S ribosomal subunit (149-152).

(ii) M_A- and M_U- factors might represent the protein component of cytoplasmic mRNP (messenger ribonucleoprotein complex), found in mammalian systems (153). It is conceivable that the protein moiety of the mRNP is responsible for specific recognition of the mRNA moiety by the ribosomes.

Unfortunately, present techniques and methodology do not permit an extensive examination of these two remaining possibilities. Until the necessary systems are established, correlation of M_A- and M_U- factors with either an S1-like protein or cytoplasmic mRNP remains speculation.

CHAPTER 2

MATERIALS AND METHODS

I. Materials

(i) Chemicals: [^{3}H]L-phenylalanine (specific activity 6.0 Ci/mmole and 6.4 Ci/mmole), [^{14}C]-amino acids (50 mCi/mmole), [^{3}H]-cytidine 5'-triphosphate (19 Ci/mmole), [^{3}H]-adenosine 5'-triphosphate (19 Ci/mmole), and [^{3}H]-polyribouridylate (8.1 mCi/mmole of polyribonucleotidyl phosphorus) were from Schwartz BioResearch. [^{3}H]L-phenylalanine (5.0 Ci/mmole), [^{3}H]L-lysine (5.0 Ci/mmole), [^{3}H]-polyriboadenylate (1 Ci/mmole of polyribonucleotidyl phosphorus) and [^{3}H]-polyribouridylate (13.7 Ci/mmole of polyribonucleotidyl phosphorus) were purchased from New England Nuclear. Unlabelled polyribouridylate and polyriboadenylate (average molecular weight 200,000 daltons) were from Miles Laboratories.

DEAE-cellulose (exchange capacity 1.20 meq/ml and 0.91 meq/ml) was purchased from Whatman Industries and Schleicher & Schuell, Inc. Phosphocellulose (exchange capacity 0.99 meq/ml) was purchased from Schleicher & Schuell, Inc. Sephadex G-200 (particle size 40-210 μ) and Sepharose 6B (particle size 40-210 μ) were purchased from Pharmacia Fine Chemicals. Trizma base, Norit A, deoxycholate and dextran sulfate were obtained from Sigma Chemical Co. Polyethylene glycol 6000 was from J.T. Baker Chemical Co. Ammonium sulfate and sucrose were of ultra pure quality, special enzyme grade and obtained from Schwartz/Mann Co. 2,5-diphenyloxazole (PPO), and 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP), were purchased from New England Nuclear Co.

The materials for polyacrylamide gel electrophoresis were purchased from BioLab. All other chemicals were of reagent grade from J.T. Baker Chemical Co., and used without further purification.

(ii) Biological Materials: Liver tissue and reticulocyte cells were obtained from New Zealand White rabbits. Heparin and marker proteins (thyroglobulin, glucose oxidase, ovalbumin, and cytochrome c), were purchased from Sigma Chemical Co. Succinyl-CoA synthetase was a gift from Dr. Pierre Pearson. Pancreatic deoxyribonuclease I (DNase I), and Pancreatic ribonuclease A (RNase A), were purchased from Worthington. Unfractionated yeast transfer RNA was obtained from Calbiochemical Co.

II. Buffer Systems

The following buffer-salt solutions were used as required:

(i) Buffer L: 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoethanol, and 0.25 M sucrose.

(ii) Buffer TR1: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 10% glycerol.

(iii) Buffer TE1: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol, and 0.1 mM EDTA.

(iv) Buffer TR2: 25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 10% glycerol.

(v) Buffer RS2: 25 mM Tris-HCl (pH 7.5), 1 mM β-mercaptoethanol, and 10% glycerol.

(vi) Buffer RS3: 12.5 mM Tris-HCl (pH 7.5), 0.5 mM β-mercaptoethanol, and 10% glycerol.

(vii) Gel Buffer: 50 mM sodium phosphate)pH 7.1), and 0.1% SDS.

III. Methods

(i) Cold Trichloracetic Acid (TCA) Wash Procedure: To remove all cold, acid-soluble material from filter discs, the discs were allowed to stand in cold 10% TCA for 40 minutes. After this they were washed four times with cold 5% TCA, each disc for 15 minutes. The discs were subsequently washed with ether-ethanol (1v:1v) and dried with ether (154).

(ii) Hot Trichloracetic acid (TCA) Wash Porcedure: To remove all hot acid soluble material from filter discs the discs were allowed to stand in cold 10% TCA for 40 minutes. After this they were washed two times with cold 5% TCA. Room temperature 5% TCA was added and the discs were placed on a hot plate at 90°C for 40 minutes. The time is critical in this latter treatment as exposure of the discs to hot TCA for greater than 40 minutes causes damage. After the hot TCA treatment, the discs were washed with room temperature 5% TCA, ether-ethanol, and finally ether (155).

(iii) Pretreatment of Nitrocellulose Filters: For the binding assays by Millipore filtration it is essential to pretreat the Millipore filters as specific below. Failure to pretreat the filters results in nonspecific retention of [^{3}H]-poly-U and in particular, [^{3}H]-poly-A. The filters were pretreated by being immersed in distilled water at room temperature, transferred to a 0.1 N NaOH solution and allowed to stand for 10 minutes (this timing is critical). The

filters were then rinsed ten times with distilled water, transferred to a 0.1 N HCl solution and allowed to stand for 10 minutes. The filters were finally rinsed ten times with distilled water and allowed to dry at room temperature. A prolonged treatment with NaOH increases the retention of [^{3}H]-poly-A; therefore special precaution must be taken to avoid soaking the filters in alkali longer than ten minutes. An acid wash alone does not reduce the nonspecific retention of poly-A.(156).

(iv) Measurement of Radioactivity: The radioactivity retained on each filter disc was measured in toluene scintillation fluid (6 g PPO and 0.5 g POPOP per litre of toluene) (154), with a Beckman LBS-230 liquid scintillation counter system. Under the conditions employed, the counting efficiencies using Whatman 3 MM filter discs and nitrocellulose Millipore filters were 2% and 20%, respectively.

(v) Estimation of RNA and Protein: RNA and protein content of the purified ribosomes were estimated by the orcinol method (purified yeast transfer RNA as a standard) and the Lowry method (bovine serum albumin as a standard) (157), respectively.

Protein concentration was determined by absorbance at 280 and 260 nm with a Bausch and Lomb spectronic 505 and corrected for nucleic acid content using the Layne table (158).

(vi) Hydrolysis of Aminoacyl Groups on tRNA prepared from rabbit liver:

To remove the aminoacyl moiety from any aminoacyl-tRNA contaminating the tRNA population, the tRNA prepared as described in Chapter 3 was subjected to mild alkaline treatment as follows (154). The tRNA was diluted in 50 mM Tris-HCl (pH 9.5) containing 1 mM $MgCl_2$ to give a final

tRNA concentration of 2 mg/ml. This solution was incubated at 37°C for 1 hour after which the tRNA was precipitated with 2 volumes of absolute methanol. After standing overnight in the cold, the precipitate was collected by centrifugation. The precipitate was then washed twice with a methanol-salts mixture (methanol:1 M NaCl:1 M $MgCl_2$ = 8:2:0.01 by volume), and allowed to stand overnight in the cold. The washed tRNA precipitate was collected by centrifugation. The resulting pellet was then dissolved in 50 mM potassium acetate buffer (pH 5.4), to a final tRNA concentration of 10 mg/ml, and stored at -20°C until required.

(vii) Treatment of Rabbit: Reticulocyte cells were obtained from New Zealand white rabbits (2 to 3 Kg) which had been injected daily with 1 ml injections of 2.5% phenylhydrazine-HCl for five days. After a two-day rest period, the rabbits were anesthetised by injecting 2 ml of Nembutal (50 mg/ml) through a marginal vein of the ear. Heparin (2 ml of a 1% solution) was also injected, and the blood was then collected. The degree of reticulation, determined by microscopic examination of stained cells, was approximately 85%.

The livers were excised from the freshly bled rabbits, chilled on ice and either used immediately (for the preparation of ribosomes), or stored at -20°C (for the preparation of tRNA).

(viii) Preparation of Reticulocyte Enzyme Mixture: A protein fraction containing aminoacyl-tRNA synthetases and other factors necessary for peptide synthesis was prepared as follows (159). Rabbit reticulocyte cells were lysed in a hypotonic solution. The supernatant obtained by differential centrifugation of the post-mitochondrial supernatant at

105,000 x G for 60 minutes, twice, was adjusted to 66% in ammonium sulfate. The resulting precipitate was collected by centrifugation, and dissolved in Buffer RS3. The protein thus prepared is completely free of RNase activity.

(ix) Partial Purification of Aminoacyl-tRNA Binding Factor: Reticulocyte cells were washed with phosphate-saline once, and lysed in hypotonic saline. Insoluble materials were removed by centrifugation at 30,000 x G for 10 minutes (fraction S30). Polyribosomes were collected from fraction S30 by preparative centrifugation at 142,000 x G for 20 minutes. The aminoacyl-tRNA binding factor was isolated from this polysomal pellet by the 1 M KCl wash method (160). In brief, the polysomes were suspended in Buffer TE1; KCl was then added to the suspension to a final concentration of 1 M. The suspension was left in the cold for 12 hours and then clarified by centrifugation at 142,000 x G for 2 hours in an SW41 swinging bucket rotor. The upper 4/5 of the supernatant was collected by aspiration. The protein in this fraction was precipitated by adding solid ammonium sulfate to saturation at 4°C. It was then stored at -20°C until used. When required, the polysome-wash fraction was dialyzed against Buffer TR1 to reduce the salt concentration, and then loaded onto a DEAE-cellulose column. The column was washed with Buffer TR1 until the $A_{280\ nm}$ recording reached the base line. The column was then washed with Buffer TR1 containing 75 mM KCl. The proteins eluting at this salt concentration were precipitated by overnight dialysis against saturated ammonium sulfate in Buffer TR1. The precipitate was collected by centrifugation at 10,000 x G for

15 minutes, dissolved in a minimum volume of Buffer TR1 containing 50% glycerol, and stored at -20°C. This fraction contains a protein factor(s) which binds with aminoacyl-tRNA but does not to mRNA. The factors which bind with mRNA elute at a much higher salt concentration and is described in detail in the text of this thesis.

(x) Preparation of [^{3}H]-lysyl-tRNA and [^{3}H]-phenylalanyl-tRNA: Individual aminoacyl-tRNA synthetases were purified according to the method described in Chapter 4. Each aminoacyl-tRNA was synthesized using either mixed yeast tRNA or rabbit liver tRNA. In short, a 10 ml reaction mixture contained 100 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 2 mM adenosine 5'-triphosphate, 400 µCi [^{3}H]L-lysine or -phenylalanine, 10 mg tRNA (yeast or rabbit liver), and 500 µg of the appropriate aminoacyl-tRNA synthetase. The reaction was initiated by the addition of tRNA at 37°C. After 30 minutes the reaction mixture was first chilled on ice and then reduced in pH to 5.4 with 1 N CH_3COOH and 1 M potassium-acetate buffer (pH 5.4). An equal volume of water-saturated phenol was then added to the mixture. After vigorous shaking, the phenol was separated by centrifugation at 10,000 x G for 15 minutes. The aqueous layer containing the aminoacyl-tRNA, was extracted an additional three times with phenol. No precipitate at the phenol-water interface was observed after this treatment signifying the removal of essentially all undesired components. The final aqueous layer was made 0.1 M in NaCl and 0.01 M in $MgCl_2$. Two volumes of abolsute methanol were then slowly added to the aqueous layer with stirring, and the mixture allowed to stand in the cold overnight. The precipitate,

representing the aminoacyl-tRNA, was collected by centrifugation at 10,000 x G and washed three times with ether-ethanol (1v:1v). The pellet was finally suspended in ether, dried, and dissolved in 50 mM potassium acetate buffer containing 50% glycerol. This final fraction was stored at -20°C. Under these conditions approximately 60% of the total cpm input was recovered in the form of [^{3}H]-aminoacyl-tRNA.

(xi) Assay of Amino Acid Acceptor Activity of tRNA: The following assay system was employed to measure the ability of tRNA to accept amino acids. A 0.5 ml reaction mixture contained 100 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 2 mM adenosine 5'-triphosphate, 10 µCi [^{3}H]-amino acid, (or 1 µCi [^{14}C]-amino acid), 50 µg protein from the aminoacyl-tRNA synthetase fraction, and 500 µg tRNA (yeast or rabbit liver). The reaction was initiated by the addition of tRNA at 37°C. At intervals, 100 µl samples were withdrawn and placed on filter discs. The discs were processed using the cold TCA method as described in Section III, (i). The radioactivity retained on each filter disc was measured as specified in Section III (iv).

(xii) Assay of AMP- and CMP-incorporating activity of tRNA: tRNA 3'-terminal nucleotidyl transferase was obtained from E. coli B by the method of Igarashi and McCalla (161). A 0.5 ml reaction mixture contained 50 mM glycine (pH 9.5), 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 µCi [^{3}H]-ATP or [^{3}H]-CTP, 150 µg tRNA (yeast or rabbit liver), and 10 µg of the purified E. coli enzyme. The reaction took place at 37°C. At intervals, 100 µl samples were withdrawn and placed on filter discs. The discs were then processed using the cold TCA wash method (Section III (i)). The

radioactivity retained on each disc was measured as described in Section III (iv).

(xiii) Assay for Aminoacyl-tRNA Synthetase: The activity of the phe-tRNA synthetase was measured at optimum conditions for aminoacylation as defined in Chapter 4. Each reaction mixture contained in 0.2 ml, 100 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 2 mM ATP, 0.2 mg unfractionated tRNA (yeast or rabbit liver), 1 mM β-mercaptoethanol, 0.5 μCi [^{3}H]L-phenylalanine, and 0.8 μg of purified synthetase. The reaction took place at 37°C and at 2 minute intervals, 80 μl aliquots were withdrawn and placed onto filter discs. The discs were washed with cold TCA as described in Section III (i), and the radioactivity retained on each measured, as specified in Section III (iv).

(xiv) Assay of Cell-Free Protein Synthesis: Each 0.5 ml reaction mixture contained 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 30 mM KCl, 0.2 mM β-mercaptoethanol, 2 mM adenosine 5'-triphosphate, 0.1 mM guanosine 5'-triphosphate, 0.1 mM phosphoenolpyruvate, 2 μCi of [^{3}H]L-phenylalanine, 100 μg of tRNA (yeast or rabbit liver), 100 μg of polyuridylate, 100 μg of purified ribosomes, and 10 μg of the reticulocyte post-ribosomal protein mixture (see III (vii)). The reaction was initiated by the addition of tRNA at 37°C. At intervals, 0.1 ml aliquots of the reaction mixture were withdrawn and placed onto filter discs. The discs were processed using the hot TCA wash method as described in Section III (ii). The radioactivity retained on each disc was subsequently measured (Section III (iv).

(xv) Assay Methods for Complex Formation of (1) mRNA:M-factor, (2) mRNA:M-ribosome:factor, and (3) Aminoacyl-tRNA:aminoacyl-tRNA binding factor:mRNA:ribosome:M-factor. Two methods were used in the binding studies: the Millipore filtration method and the sucrose density gradient centrifugation method. With the Millipore filtration method, it is possible to detect mRNA binding to crude ribosomes or mRNA binding factors since both ribosomes and the factors will be retained on the filter, and since the free mRNA will not be retained. Therefore, the retention of [^{3}H]-mRNA on the filter in the presence of ribosomes or factors represents complex formation with mRNA. The same principle can be applied to the complex formation of [^{3}H]-aminoacyl-tRNA with the aminoacyl-tRNA binding factor as it also will be retained on the filter. The Millipore method, however, cannot be used to detect the complex formation between mRNA:ribosome:M-factor, or the complex formation between ribosomes:M-factors:aminoacyl-tRNA:aminoacyl-tRNA binding factor because the mRNA binding factor will be retained in the absence of ribosomes, as stated above. The latter two complex formations therefore had to be analyzed by the sucrose density gradient method. Keeping these limitations in mind, the Millipore assay method was employed whenever possible.

Millipore assay: The reaction mixture contained in 0.5 ml, 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, and the desired combination and amount of [^{3}H]-polyribonucleate, [^{3}H]-aminoacyl-tRNA and binding factor fraction (aminoacyl-tRNA and/or mRNA). In any reaction involving the binding of aminoacyl-tRNA, 0.1 mM GTP was added to the

reaction mixture. The various combinations of the reaction components for individual experiments are described in Chapter 6. The reaction was allowed to proceed on ice for 5 minutes, and stopped by diluting each sample with 5 ml of a buffer containing buffer and salts in similar proportions to those of the reaction mixture. The diluted sample was then filtered through pretreated Millipore filters (Section III (iii), and rinsed five times with 2 ml of the same buffer. The filters were dried for 5 minutes in an 85°C oven, and examined for the retention of radioactive material as described in Section III (iv).

Sucrose Density Gradient Analysis: The sucrose density gradient method allows separation of polyribosomes, monomeric ribosomes, ribosomal subunits, protein factors, and mRNA. It should be pointed out however, that this method limits the number of samples to be processed each time to the rotor capacity (6 tubes in the SW41 swinging bucket rotor). In addition, only samples which require the same duration of spin can be processed at one time. Despite these limitations, the sucrose density gradient centrifugation method is at present the only method which allows us to analyze the complex formations of mRNA-ribosome:mRNA binding factor, or mRNA:ribosome:mRNA binding factor:aminoacyl-tRNA:aminoacyl-tRNA binding factor. The reaction mixture for each binding assay was the same as that described for the Millipore filter assay. The 0.5 ml reaction mixture was applied onto 12 ml of preformed sucrose density gradient (8-15% in suitable buffer), and spun at 37,000 rpm in an SW41 rotor operated in a Beckman L3-50 ultracentrifuge. The durations of the spin were 60 minutes for the observation of polysome formation, and 180

minutes for the separation of monomeric ribosomal-complex from free protein or RNA. After ultracentrifugation, 6-drop fractions were collected from the bottom of each tube with a piercing device. The drops were either collected directly onto filter discs or into test-tubes. The former method of collection was employed when radioactivity was to be measured. For the detection of radioactivity, the discs were allowed to dry at room temperature and the radioactivity measured as described in Section III (iv). For the detection of ultraviolet absorbing material, fractions contained in test-tubes were diluted with an equal volume of distilled water. The absorbance of each fraction at 260 nm was then measured using a Bausch and Lomb Spectronic 505.

(xvi) Assay of RNase Activities: The RNase activities of various fractions was monitored by measuring the hydrolysis of radioactively-labelled polynucleates (162). In short, a 0.5 ml reaction mixture of Buffer L contained the appropriate [^{3}H]-polyribonucleate and the fraction to be tested. RNase A was included in the control experiment. The reaction occurred at 37°C and at intervals, 0.1 ml aliquots were removed and placed onto filter discs. The discs were washed with cold TCA as described in Section III (i), and the radioactivity retained on each filter was measured as described in Section III (iv).

(xvii) Analytical Ultracentrifugal Analysis: Sedimentation velocity patterns were measured in a Beckman Model E analytical ultracentrifuge. Samples were diluted to A_{260}=/nm/ml in 0.1 M KCl solution. The sample solution was loaded into a Kel-F 40-12 mm centerpiece cell with quartz windows, and spun in a AN-D type aluminum rotor at 56,000 rpm (for rabbit

liver tRNA) or 40,000 rpm (for rabbit liver ribosomes) at 20°C. Photographs were taken at 265 nm at either 8 minute intervals (for rabbit liver tRNA) or 2 minute intervals (for rabbit liver ribosomes) after the speed was attained.

(xviii) Acrylamide Gel Electrophoresis: Polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborne (163). Gels (0.6 cm x 10 cm) consisted of 7% acrylamide, the corresponding concentration of N,N'-methylene-bis-acrylamide, 50 mM sodium phosphate (pH 7.1), and 0.1% SDS. Polymerization was catalyzed by N,N,N',N'-tetramethylenediamine and ammonium persulphate at final concentrations of 0.03% and 0.07%, respectively.

Protein samples were dialyzed for 16 hours against 50 mM sodium phosphate, pH 7.1. Protein samples (6-12 µg) were denatured by heating to 80°C for 20 minutes in 50 mM dithiothreitol and 2.0% SDS. Samples were made 20% in glycerol by the addition of 50% glycerol. Bromphenol blue (3 µl of a 0.05% solution) was also added for tracking. Samples were then applied to the gels by layering beneath the electrophoresis buffer (50 mM sodium phosphate, pH 7.1, containing 0.1% SDS), and electrophoresed at room temperature for 2.5 hours at 6 milliamps per gel. Gels were stained at 37°C for 2 hours in 9.2% acetic acid, 45% methanol, and 0.25% Coomassie brilliant blue. Destaining was done in 7.5% acetic acid and 45% methanol using an ISCO horizontal rapid destainer. Gels were stored in 7.5% acetic acid and kept in the dark. Densitometric tracings of stained gels were performed with an ISCO Gel Scanner (Model 659).

Molecular weights were determined from SDS-acrylamide gel electrophoresis by comparing the migration distances of the unknown proteins with those of known molecular weight proteins. Swelling of the gels during staining was corrected for by the use of the following correction factor:

$$\text{Mobility} = \frac{\text{protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{dye migration}}$$

Succinyl-CoA synthetase, a protein with a known $\alpha_2\beta_2$ subunit structure (α = 29,500, β = 38,700) was routinely run on SDS-acrylamide gels to ensure the reliability of the method. Densitometric tracings of such gels showed rise to two distinct bands. Comparison of the mobilities of these two bands with those of the marker proteins indicated a value of 29,000 for the smaller subunit, and 39,000 for the larger subunit, in agreement with the accepted value (Dr. Pierre Pearson, personal communication).

(xix) Molecular Weight Determination by Molecular Sieving Column Chromatography: Molecular weights were determined from either Sephadex G-200 or Sepharose 6B columns (150 ml bed volume) by comparing the elution position of the unknown proteins with those of known molecular weight. The column was first equilibrated with the

appropriate buffer (as specified in text) containing 150 mM ammonium sulfate until the baseline at 280 nm was attained on a UV monitoring system (ISCO). The sample (0.8-1.2 ml) containing 0.24 M sucrose was then applied to the column and developed with the appropriate buffer containing 150 mM ammonium sulfate.

CHAPTER 3

LARGE SCALE PREPARATION OF RABBIT LIVER tRNA

I. Introduction

As mentioned in Chapter 1, analysis of the mechanism of mRNA:ribosome complex formation required two basic approaches. The first was to demonstrate mRNA:ribosome complex formaltion under the appropriate conditions. The second was to ensure that the complex formation was a biologically significant process. The specific binding of aminoacyl-tRNA to the mRNA:ribosome complex, being the next logical step in the initiation of protein synthesis was chosen as a criterion for biological significance. The prerequisite for the second approach was the preparation of [^{3}H]-aminoacyl-tRNA. The first task, therefore, was to obtain a sufficient amount of intact tRNA - possibly a gram quantity, to conduct our experiments. In addition, on the basis of preliminary experiments, we realized that yeast tRNA was an ineffective substrate for the liver system; therefore we decided to prepare homologous rabbit liver tRNA.

When we first set out to prepare rabbit liver tRNA in 1970, only one method for the preparation of tRNA from mammalian tissues was reported (169). Application of the isolation procedure originally devised for bacterial systems (164-166) usually resulted in a low yield of pure tRNA (167). The large amount of non-nucleic acid material in the homogenate of mammalian tissues made the extraction of nucleic acid very inefficient. As a result progress in defining the physico-chemical and biological properties of mammalian tRNA's was delayed. For example, as of 1970,

no mammalian tRNA had been purified to homogeneity; as a result of this, the complete base sequence of tRNA was not deduced. It was only in 1972 that the sequence of rat liver serine acceptor tRNA was worked out (168).

The difficulties in isolating mammalian tRNA employing the procedures devised for bacterial cells were partially overcome by the introduction of isopropanol fractionation followed by DEAE-cellulose chromatography (165, 169-173). When applied to the rabbit liver system, however, this method gave a final yield of tRNA only one-tenth of that obtained in the beef liver system. To obtain rabbit liver tRNA, modification of existing methods was required.

The preparation of gram quantities of rabbit liver tRNA is described in this chapter. The method includes: phenol treatment of the liver during the homogenization step, extraction of the phenol layer with water, isopropanol fractionation of the aqueous layer, ultracentrifugation of the tRNA containing fraction, and purification of tRNA on DEAE-cellulose. The principle modification is ultracentrifugation of the isopropanol fraction. This step substantially reduces the content of alcohol precipitable impurities, in particular polysaccharides. This contaminant behaves like DNA and if not removed, interferes with subsequent purification of the tRNA. One advantage of this new method is that phenol treatment of the liver during the homogenization step and subsequent extraction of the phenol layer with water permits the separation of tRNA from the bulk of high molecular weight RNA (ribosomal RNA) (174). The cause for this

selective extraction of tRNA is not known at present. The method described in this chapter can be applied to nonfasting rabbits and results in a high yield of pure tRNA's capable of accepting all amino acids.

II. Results

(i) Procedure for the Isolation of Rabbit Liver tRNA: Rabbit liver was obtained as described in Chapter 2, Section III (vii). 500 g of frozen rabbit liver was minced and homogenized in 1000 ml of a solution containing 5 mM EDTA, Norit A (0.1% w/v), and phenol (50% v/v), adjusted to pH 8.0 with 0.1 N NaOH. As noted by Robison and Zimmerman with the beef liver system (175), the absence of added salt in the phenol extraction step is important in minimizing the amount of DNA which coextracts from the tissue. Inclusion of Norit A as a nonspecific adsorbant of protein reduces nuclease activities in the tRNA containing fraction. Homogenization was carried out in a 2 gallon stainless-steel Waring Blender (Model CB-5) operated at minimum speed for 30 seconds. The resulting homogenate was then centrifuged at 6000 x G for 30 minutes and the pale-yellow, aqueous layer, containing the tRNA was collected by aspiration. In order to ensure maximum recovery the interface region was separately collected, centrifuged, and combined with the above tRNA fraction. Solid potassium acetate was then added to the aqueous layer to a final concentration of 300 mM. The pH of this resulting solution was approximately 6.5.

A differential precipitation procedure by isopropanol was then performed. First, 0.25 volumes of isopropanol was slowly added to this

solution with stirring. This preliminary step removed a large part of the alcohol precipitable impurities. The precipitate at this concentration of isopropanol was removed by centrifugation at 8,000 x G for 30 minutes, and discarded. An additional 0.95 volumes of isopropanol was added to the supernatant. Again Norit A was included as a safeguard against residual nuclease activity. The precipitate containing the tRNA was collected by centrifugation. This pellet was suspended in 100 ml of 50 mM potassium acetate (pH 5.4) containing 140 mM NaCl. The resulting solution was clarified by centrifugation at 12,000 x G for 10 minutes. The supernatant was loaded into a Beckman type 42 fixed-angle rotor and was centrifuged at 124,000 x G for 180 minutes. After centrifugation, the clear supernatant was collected and used for subsequent purification of tRNA. The clear, copious pellets, comprising approximately 2/3 of the total volume of combined supernatant and pellet, contained predominantly polysaccharide and were discarded.

To ensure the complete removal of polysaccharide and residual protein, the tRNA was further purified on a DEAE-cellulose column (3 cm x 100 cm). The supernatant fraction obtained by ultracentrifugation was loaded onto a column which had been equilibrated with 50 mM potassium acetate buffer (pH 5.4). The column was then washed with the buffer containing 400 mM NaCl until the base line of the UV-monitoring system (ISCO) at 254 nm was attained. This initial wash removed the residual protein and polysaccharide contaminants. The tRNA was finally eluted with the buffer containing 1.5 M NaCl. The effluent, approximately 100 ml, was diluted with glass-distilled water to give a final concentration

of 1.0 M NaCl. In order to precipitate the tRNA, 1.5 volumes of isopropanol was slowly added to this solution with stirring. To ensure complete precipitation of tRNA, the fraction was allowed to stand overnight at 4°C. The tRNA precipitate was subsequently collected by centrifugation at 10,000 x G for 10 minutes. At this stage of purification, the average yield of tRNA as sodium-salt was approximately 40 mg/100 g of rabbit liver.

Since preparations of tRNA often contain tRNA in the acylated form (176), a deacylation step involving mild alkaline treatment was incorporated into the rabbit liver tRNA purification scheme as specified in Chapter 2, Section III (vi). The final tRNA solution (10 mg/ml) in 50 mM potassium acetate buffer (pH 5.4) was stored at -20°C. Rabbit liver tRNA prepared in this manner is stable for at least two years as determined by its ability to accept [^{3}H]L-phenylalanine. A summary of the optical properties and yield in terms of A_{260} units during the preparation is presented in Table 3.

(ii) Sedimentation Velocity of tRNA In order to examine the homogeneity of tRNA prepared by the method described above, the sedimentation velocity boundary profile of the tRNA was measured in a Beckman model E analytical ultracentrifuge as described in Chapter 2, Section III (xvii). Photographs were taken at 265 nm at 8 minute intervals after the speed (56,000 rpm), was attained. The densitometric tracing of the photographs is presented in Fig. 2. The tRNA sedimented as a single moving boundary with no evidence of other sedimenting materials. From this profile, the sedimentation coefficient was calculated to be

TABLE 3

Optical Properties of tRNA Fraction during the Purification Procedure

Fraction	λ_{max}	A_{260}/A_{280}	Total A_{260} Units
Isopropanol (0.95 vol) fraction	No max*	0.95	64,000*
Supernatant after ultracentrifugation	275	1.65	11,000
Effluent from DEAE-cellulose (1.5 M NaCl)	257	3.04	4,500
Final tRNA	257	3.00	3,500

* No distinct absorption maximum is seen between 240 and 320 mμ so that the total A_{260} units do not reflect the content of nucleic acids in this fraction.

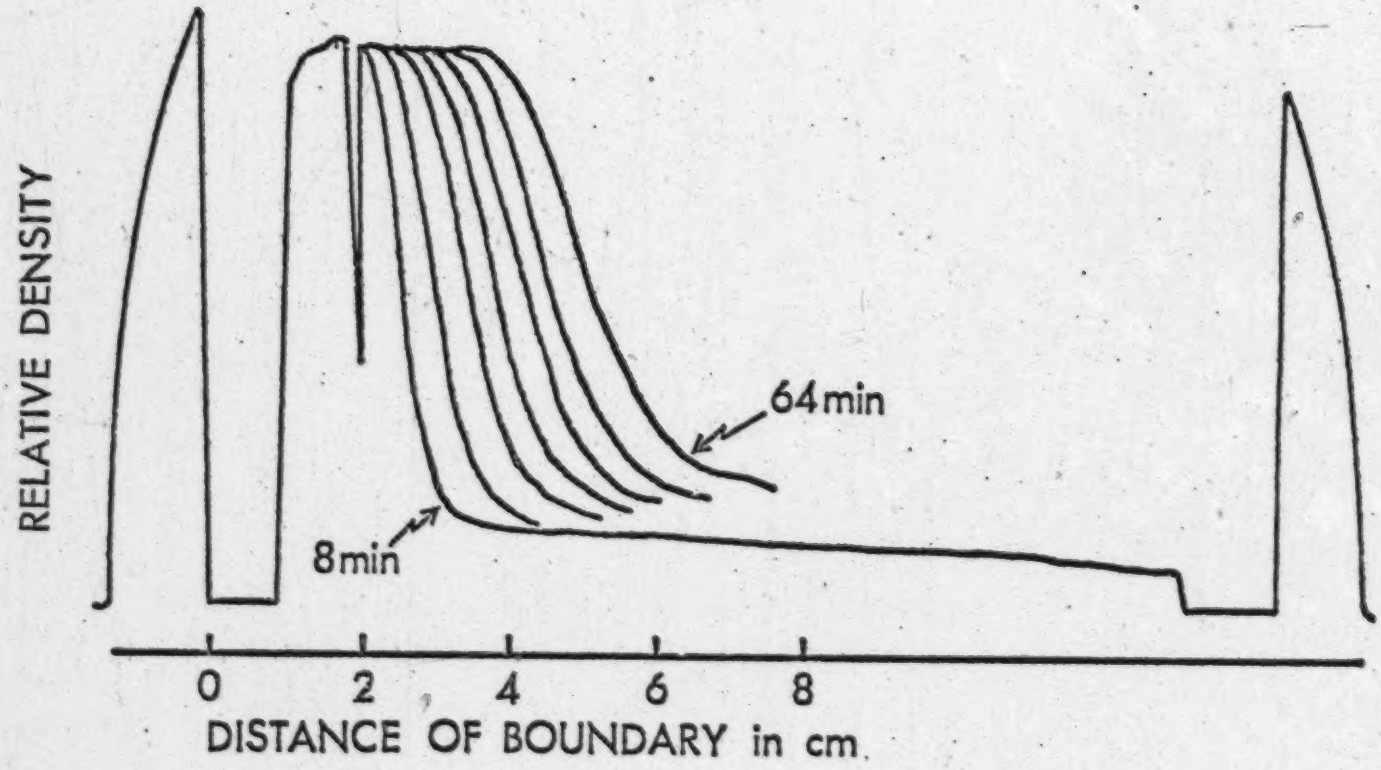


FIGURE 2: Sedimentation velocity pattern of rabbit liver tRNA in 0.1 M KCl, determined in a Spinco Model E centrifuge at 56,000 r.p.m. at 20°C. The photographs were taken at eight min intervals after the speed was attained. The details of the method are presented in Chapter 2, Section III (xvii).

4.15 ± 0.05 S. This value is in close agreement with the sedimentation coefficient of human placenta tRNA (167).

(iii) Intactness of the 3'-terminal -CCA Sequence of tRNA: In the preparation of tRNA from beef liver, Robison and Zimmerman found that a large percentage (37%) of the tRNA molecules lacked the 3'-terminal adenylate residue. This property caused an inefficiency in amino acid acceptance by the tRNA. Moreover, they detected the stimulation of the aminoacylation reaction by added CTP. This fact indicated that a fraction of tRNA molecules prepared by their method also lacked the adjacent CMP residue(s) (167). The deficiency of 3'-terminal nucleotide residues in these studies was attributed in part to the nucleolytic process which occurred during the slow freezing of the liver after its removal from the animal. In order to see whether or not a similar situation existed for the tRNA prepared from rabbit liver (Section (i)), the efficiency of the tRNA as a receptor molecule in the 3'-terminal nucleotidyl transferase reaction was examined as described in Chapter 2, Section III (xii). This enzyme is known to specifically regenerate the -CCA sequence at the 3'-terminal position of a partially degraded tRNA from any source (161). When the prepared tRNA was assayed for AMP incorporation into the 3'-terminal using the E. coli transferase, no incorporation was observed. This suggested that little, if any, of the prepared tRNA lacked the 3'-terminal adenylate residue, or that the tRNA lacked more than the first two terminal residues. In order to determine which of the two alternatives was correct, a similar experiment testing CMP incorporation was performed. The results indicated that

some of the tRNA lacked the 3'-terminal cytidylate residue(s) adjacent to the adenylate residue. However, based on the incorporation of CMP by the same concentration of snake venom treated tRNA (161), a procedure which removes the 3'-terminal -CCA sequence, the fraction of rabbit liver tRNA missing the 3'-terminal -CA or -CCA was estimated to be less than 1% (Fig. 3).

(iv) Amino Acid Acceptance of tRNA: Since the tRNA purified from rabbit liver was to be used for the preparation of [^{3}H]-aminoacyl-tRNA's for specific binding studies, it was imperative to ensure that it accepted amino acids. Because different aminoacyl-tRNA's were to be prepared for later specific binding studies, it was also important to ensure that the tRNA preparation contained a full complement of tRNA's (one for each of the 20 common amino acids). In order to determine the distribution of different amino acid-specific tRNA's, each preparation of tRNA was examined for its ability to accept 15 amino acids. The method employed is described in Chapter 2, Section III (xi). The amino acid acceptance of rabbit liver tRNA was compared with that of a reference stock of commercially available yeast tRNA. The results obtained for such an experiment are presented in Table 4.
Due to the use of radioactively labelled amino acids as substrates, the results in Table 4 do not represent the saturation level of a given tRNA for its specific amino acid. Since phenylalanine accepting tRNA was for immediate use, the saturation level of tRNA specific to phenylalanine was determined (Fig. 4). An increasing amount of nonradioactive phenylalanine was added to a reaction mixture

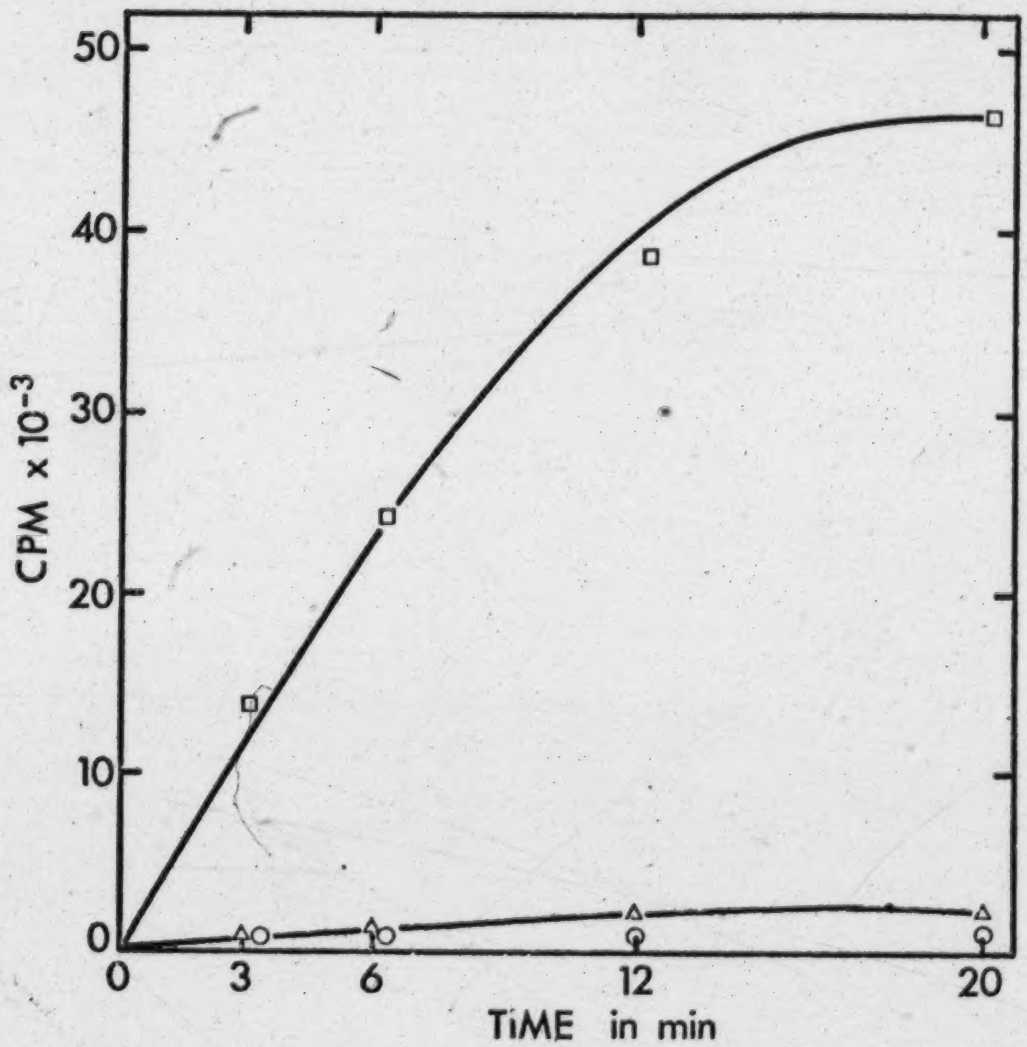


FIGURE 3: tRNA 3'-terminal nucleotidyl transferase reaction with prepared rabbit liver t RNA. The reaction was carried out as described in Chapter 2, Section III (xii).

CMP acceptance by snake-venom treated tRNA (□).

CMP acceptance by prepared rabbit liver tRNA (Δ).

AMP acceptance by prepared rabbit liver tRNA (O).

TABLE 4

Amino Acid Accepting Activity of Prepared Rabbit Liver tRNA

^{14}C-amino acid*	µµmole amino acid incorporated per mg rabbit liver tRNA	µµmole amino acid incorporated per mg yeast tRNA
Alanine	377	0
Arginine	822	0
Aspartic Acid	44	0
Cystine	144	66
Glycine	133	0
Histidine	288	244
Isoleucine	155	0
Lysine	544	211
Methionine	377	200
Phenylalanine	577	155
Proline	266	33
Serine	700	233
Threonine	900	233
Tyrosine	655	366
Valine	588	100

The results were expressed as µµmoles of amino acid incorporated at 10 min after initiation of reaction.

* ^{14}C-amino acid (50 mCi/mmole).

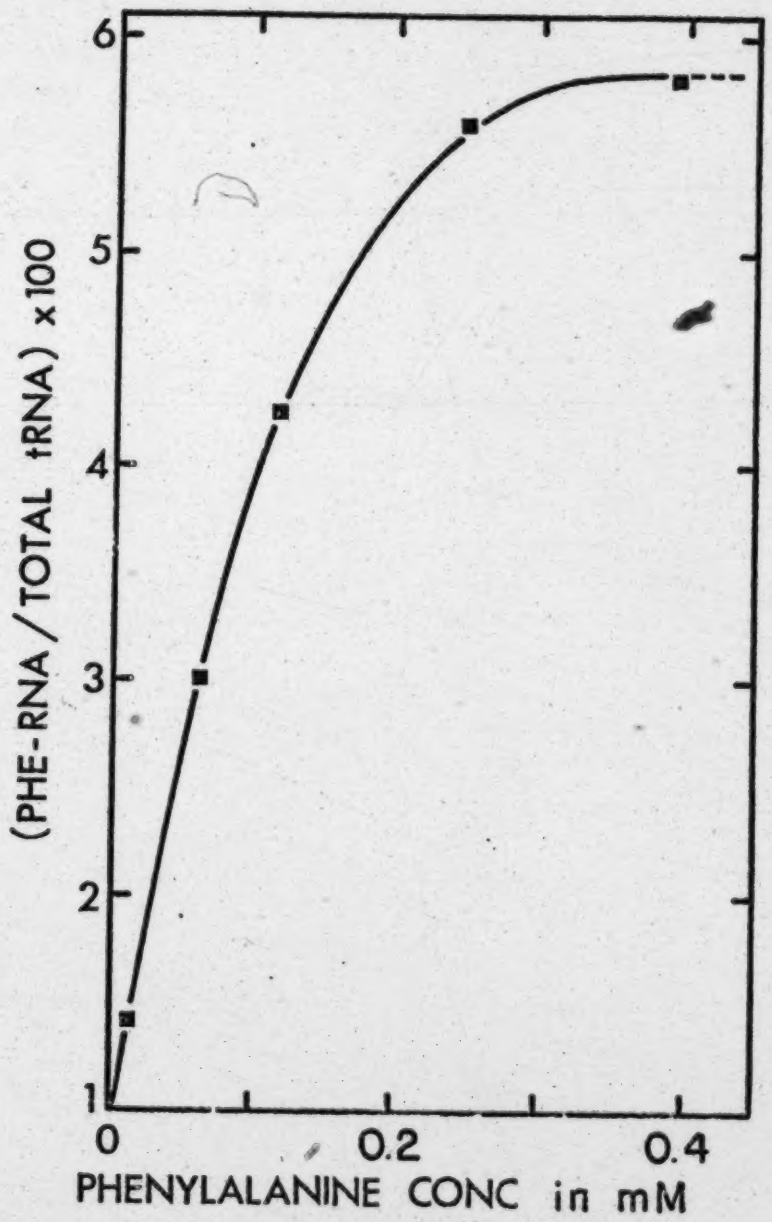


FIGURE 4: The saturation level of rabbit liver tRNA specific for phenylalanine. Reaction conditions were described in Chapter 2, Section III (xi). Phe-tRNA synthesis was examined in the presence of varied amounts of phenylalanine as described in Chapter 2, Section III (xi). The results are expressed as per cent phe-tRNA (ordinate) versus added-unlabelled phe concentration (abscissa).

containing a fixed amount of [^{3}H]L-phenylalanine (1.7 mμmoles), and the total synthesis of phenylalanine-tRNA was assayed as described in Chapter 2, Section III (xi). From these studies it was found that 5 to 6% of the purified rabbit liver tRNA was phenylalanine acceptor tRNA.

III. Discussion

The preparation of rabbit liver tRNA as described in this Chapter provides an efficient means of obtaining a large quantity of homogeneous tRNA. The resulting tRNA fraction appears to contain a full complement of amino acid acceptance activities and is therefore suitable for a variety of studies. Although the procedure makes use of a series of well-established methods for each step (177,178), strict adherence to the described sequence of applying these methods im important to ensure successful tRNA preparation from rabbit liver. The key factor in the preparation method is the inclusion of ultracentrifugal separation of polysaccharides immediately after isopropanol fractionation of the liver homogenate. Ultracentrifugation serves two functions: it substantially reduces the volumes of the tRNA containing fraction and it decreases the amount of interfering components present during the subsequent DEAE-cellulose chromatography of the tRNA fraction.

Although the prepared tRNA accepts a variety of amino acids, the degree of acceptance of the various amino acids differs. The pattern of accepting activity was reproducible with different preparations of rabbit liver tRNA and may be interpreted as a reflection of an uneven distribution of various tRNA species in rabbit liver. However, a proportional distribution of aminoacyl-tRNA synthetases in the enzyme preparation used in the

aminoacylation reaction cannot be ruled out.

One of the most striking features of the amino acid acceptance studies is the greater efficiency of rabbit liver tRNA as compared to commercial yeast tRNA. This result is in agreement with the observation by Delihas and Staehelin with rat liver tRNA (167). One explanation is that mammalian aminoacyl-tRNA synthetase has a recognition mechanism specific to homologous tRNA, such that it will distinguish a heterologous yeast tRNA. This phenomenon exists in the recognition of rabbit liver $tRNA^{met}$ by methionyl-tRNA synthetase. Three species of $tRNA^{met}$ were purified from rabbit liver by Petrissant and co-workers and were each esterified by the same rabbit liver methionyl-tRNA synthetase; however, only one species was esterified by E. coli methionyl-tRNA synthetase (179). Although species specificity might account for the difference in aminoacylation of rabbit liver tRNA and yeast tRNA, other possibilities cannot be ruled out. Whatever the reason, it is apparent that the homologous aminoacylation system is more efficient that the heterologous system, a phenomenon we anticipated at the initiation of our research. Thus, as described in this chapter, the first step toward establishment of an homologous cell-free system from rabbit liver was accomplished.

CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF RABBIT LIVER PHENYLALANYL-tRNA SYNTHETASE

I. Introduction

According to our original experimental design, the preparation of [^{3}H]-aminoacyl-tRNA's was necessary to test the biological significance of the mRNA:ribosome complex formed under cell-free conditions. As pointed out in Chapter 1, this necessitated the preparation of two components: intact tRNA and aminoacyl-tRNA synthetase. The purification of biologically active tRNA's was accomplished as described in Chapter 3. The purification of the second component, required to catalyze the aminoacylation of tRNA, is the subject of this Chapter.

Since homogeneous rabbit liver tRNA had proven to be considerably more effective than an equal concentration of commerical yeast tRNA in the aminoacylation reaction (Chapter 3, Section II (iii)), we were optimistic that sufficient amounts of [^{3}H]-aminoacyl-tRNA could be prepared with purified rabbit liver tRNA and a crude post-ribosomal protein fraction. In fact, radioactivity measurements demonstrated that the crude protein fraction was able to catalyze the formation of [^{3}H]-aminoacyl-tRNA. However, the amount of product was not sufficient to permit isolation by phenol extraction. Increasing the concentration of the crude enzyme and/or the substrates -tRNA, amino acid, and ATP- had little effect on the final yield of aminoacyl-tRNA. These results

suggested two possibilities: there was an insufficient amount of a given aminoacyl-tRNA synthetase and/or proteins other than the synthetase were interfering with the function of the synthetase. To rectify this situation, it appeared necessary to purify the synthetase from the crude protein fraction. Thus we attempted a purification of two species of synthetase required for the preparation of two aminoacyl-tRNA's: phenylalanyl-tRNA synthetase for the poly-U directed binding system, and lysyl-tRNA synthetase for the poly-A directed binding system. Of these two, phenylalanyl-tRNA synthetase was purified to homogeneity and studied in depth with regard to its reaction mechanism. In keeping with our requirement to establish a completely homologous system, the enzymes were purified from rabbit liver.

As in the case of tRNA purification (Chapter 3), there was no established method for the purification of mammalian synthetases in 1970. In fact, no mammalian aminoacyl-tRNA synthetase had been studied, although many of the aminoacyl-tRNA synthetases from E. coli had been purified to homogeneity and well characterized (15-21). It is interesting to note that at present, only three mammalian aminoacyl-tRNA synthetases have been studied in reasonable depth: phenylalanyl-tRNA synthetase from rat liver (102); seryl-tRNA synthetase from hen liver (103); and, tryptophanyl-tRNA synthetase from human placenta (104,105), and beef pancreas (180). The reason for this scarcity of information concerning mammalian synthetases appears to be the technical difficulties encountered in the isolation and maintenance of a biologically-functional, pure enzyme.

This chapter focuses on the isolation and characterization of phenylalanyl-tRNA synthetase from rabbit liver. Since the genetic code

for phenylalanine is UUU, and since the biological significance of the poly-U:ribosome complex can be examined by specific binding of phenylalanyl-tRNA to this binary complex as specified by the genetic code on the bound poly-U, phenylalanyl-tRNA synthetase was the necessary and sufficient choice. In addition, the choice of phenylalanyl-tRNA synthetase provided an excellent opportunity for comparative analysis. During the course of our studies, phenylalanyl-tRNA synthetase was isolated and characterized from E. coli (15), and yeast (181). Moreover, preliminary studies were reported for rat liver phenylalanyl-tRNA synthetase (102). Although these enzymes exhibited the same catalytic patterns - that is the activation of phenylalanine and transfer of the amino acid moiety to tRNA - they displayed significant differences in their physical and chemical properties, such as molecular weight, subunit structure, and kinetic mechanism of the reaction. Our choice of rabbit liver phenylalanyl-tRNA synthetase, therefore, allowed us to test the efficiency of our isolation procedure relative to that reported for the same enzyme from rat liver (102), and to proceed with comparative studies with the same and different aminoacyl-tRNA synthetases from both prokaryotic and eukaryotic sources.

The procedure most feasible for the purification of phenylalanyl-tRNA synthetase from rabbit liver includes conventional column chromatography with DEAE-cellulose, phosphocellulose, and Sephadex G-200. The most critical aspect of the procedure concerns the effect of Mg^{2+} concentration on the stabilization and the purification of the enzyme. The buffer system for the purification of phenylalanyl-tRNA synthetase

must contain Mg^{2+} (1-5 mM) to stabilize the enzyme as an active form of approximately 270,000. The use of a buffer containing no Mg^{2+} throughout the purification results in complete loss of enzymic activity accompanied by dissociation of the enzyme into subunits. However, continuous exposure of the enzyme to Mg^{2+} prevents the removal of the heme complex from the enzyme fraction. Thus, insertion of EDTA treatment of the enzyme fraction followed by chromatographic separation of the heme complex was introduced. This step was found to be absolutely necessary to purify the enzyme to homogeneity. The brief exposure of the enzyme to low Mg^{2+} concentration has no effect on its activity or subunit structure.

The method described in this chapter permits the preparation of homogeneous phenylalanyl-tRNA synthetase from rabbit liver. The native enzyme has a molecular weight of approximately 270,000. SDS-gel electrophoretic analysis of the synthetase indicated that it is composed of two types of subunits and is of the form $\alpha_2\beta_2$ (α = 58,000, β = 76,000).

In addition to the purification scheme and subunit structure of rabbit liver phenylalanyl-tRNA synthetase, this chapter presents results concerning the kinetic mechanism of the synthetase. These results suggested the following properties: (1) kinetic parameters of rabbit liver synthetase were found comparable to those of other synthetases. (2) Dead-end inhibitor studies, as well as Cleland analysis of initial velocity patterns provided evidence that the mode of substrate addition to the enzyme is random. (3) Aurintricarboxylic Acid (ATA), a dye known to inhibit mRNA:ribosome complex formation in E. coli (1982,183) strongly

inhibits the aminoacylation reaction catalyzed by the synthetase. The interesting feature concerning the ATA inhibition is that although ATA appears to interact directly with the synthetase, the mode of interaction appears to be non-competitive with respect to all three substrates - tRNA, amino acid, and ATP.

II. Results

(i) Purification of Rabbit Liver Phenylalanyl-tRNA Synthetase

(a) Extraction of Crude Liver Protein Factors: An overall purification scheme for rabbit liver phenylalanyl-tRNA synthetase is summarized in Figure 5 and Table 5. All procedures were performed at 0-4°C unless otherwise specified. Rabbit liver was obtained from New Zealand white rabbits as described in Chapter 2, Section III (vii). 200 grams of liver from a freshly-bled rabbit was minced and homogenized in two volumes (w/v) of Buffer L (see Chapter 2, Section II (i)). Homogenization was carried out in a one-quart, stainless steel Waring Blender (Model 5011) operated at top speed for 30 seconds. Deoxycholate was then added to the homogenate to a final concentration of 10 mM. In order to degrade the DNA released from nuclei, the homogenate was treated with DNase at a final concentration of 5 μg/ml for 20 minutes. Complete disruption of the tissue was ensured by homogenizing the deoxycholate-treated fraction with two strokes of an electrically-driven, tight-fitting, teflon homogenizer operated at medium speed. Cellular debris was removed by centrifugation at 10,000 x G for 30 minutes in a Beckman J21 centrifuge. The supernatant was collected by aspiration, diluted to 450 ml with Buffer L, and centrifuged at 100,000 x G for 120 minutes in a

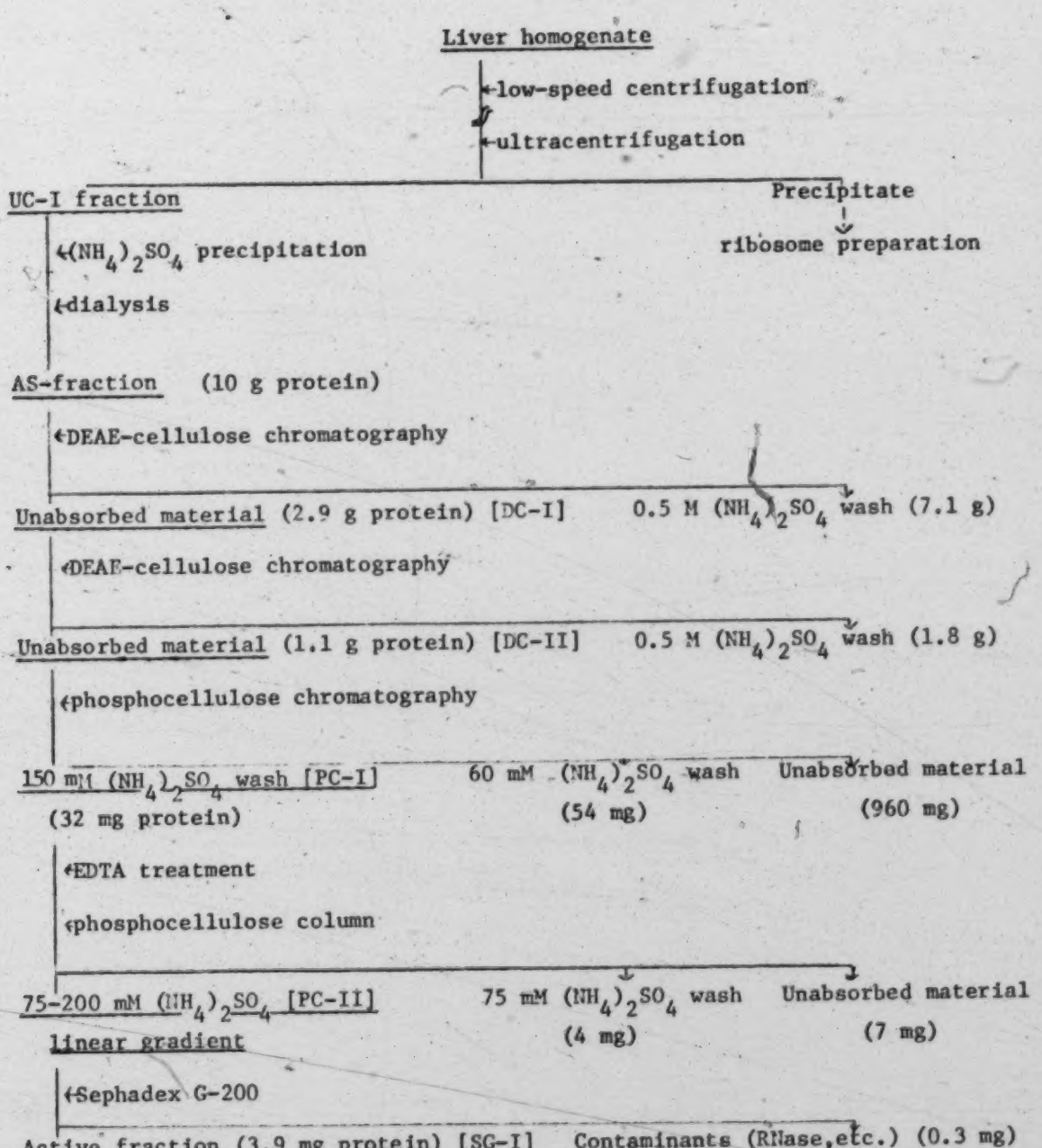


FIGURE 5: Purification Procedure for phe-tRNA Synthetase

TABLE 5

Purification of phe-tRNA Synthetase

Step	Fraction*	mg Protein	% Protein Recovered	Specific Activity (nmole phe/ mg/min)	Purification	Total Activity (μmole phe)	% Activity Recovered
1	AS-1	10,000	100	0.14	1 X	1.40	100
2	DC-1	2,900	29	0.47	3.4 X	1.36	98
3	DC-11	1,100	11	1.25	8.9 X	1.38	98
4	PC-1	32	.32	47.7	338.7 X	1.53	108
5	PC-11	4.3	.02	250.0	2333.3 X	1.08	97
6	SG-1	3.9	.015	363.6	2666.7 X	1.42	100

* See Fig. 5 for the designation of each fraction.

Type 42 fixed-angle rotor operated in a Beckman L3-50 ultracentrifuge The resulting supernatant, designated as UC-1 fraction, was used for the purification of the synthetase.

In order to precipitate the protein, solid ammonium sulfate was added slowly to UC-1 fraction with stirring. After the addition of the ammonium sulfate to saturation, stirring was continued for an additional hour. The resulting precipitate was collected by means of centrifugation at 10,000 x G for 30 minutes. The supernatant was discarded and the resulting pellet was suspended in a minimum volume of Buffer TR2 (see Chapter 2, Section II (iv)). This suspended pellet, designated as AS-fraction, could be stored at -20°C for at least two months without loss of the phenylalanyl-tRNA synthetase activity. The synthetase activity of each fraction throughout the purification was determined as described in Chapter 2, Section III (xiii).

(b) DEAE-Cellulose Chromatography of AS-fraction: A ten-gram portion of protein from AS-fraction was dialyzed against 40 times its volume of Buffer TR2 for 16 hr in order to reduce the salt concentration. When required, the dialyzed fraction was further diluted with the same buffer to ensure a monovalent cation concentration of less than 50 mM as determined by a conductivity meter. The diluted fraction was then applied to a DEAE-cellulose column (6 cm x 40 cm) which had been equilibrated with Buffer TR2. The column was first washed with 800 ml of Buffer TR2. This initial wash fraction contained the synthetase activity and was designated as DC-1 fraction. Phenylalanyl-tRNA synthetase activity was not detected in the subsequent 500 mM ammonium sulfate wash of the column, suggesting

that essentially all the enzyme activity eluted with the initial Buffer TR2 wash.

(c) Rechromatography of DC-I fraction on a DEAE-Cellulose Column: The above DC-1 fraction was immediately diluted with Buffer TR2 to reduce the monovalent cation concentration to 20 mM NH_4^+ and then applied to a second DEAE-cellulose column (2 cm x 70 cm). The column was washed in the same manner as the first DEAE-cellulose column. Again the phenylalanyl-tRNA synthetase activity was detected in the initial wash fraction. This fraction was designated as DC-II fraction. The second DFAE-cellulose chromatography achieves only a two-fold purification of the enzyme from DC-I fraction. However, this rechromatography was found to be necessary since omission of this step resulted in an ineffectual purification of the enzyme by subsequent phosphocellulose chromatography.

(d) Phosphocellulose Column Chromatography of DC-II fraction: Since the phenylalanyl-tRNA synthetase activity was not retained on DEAE-cellulose, we attempted further purification on a phosphocellulose column. DC-II fraction was immediately applied to a phosphocellulose column (2 cm x 70 cm) which had been equilibrated with Buffer TR2. The column was then washed successively with Buffer TR2 containing: no ammonium sulfate, 60 mM ammonium sulfate, 150 mM ammonium sulfate and 450 mM ammonium sulfate. The phenylalanyl-tRNA synthetase activity was detected only in the 150 mM ammonium sulfate fraction. This was designated as PC-I fraction.

At this stage of purification, two features of the enzyme fraction were noticeable: (1) up to the stage of phosphocellulose column chromatography, the inclusion of 1 mM $MgCl_2$ and 10% glycerol was required to

maintain the enzymic activity of the fractions. (2) PC-I fraction was contaminated with heme as evidenced by the red colour associated with the enzyme fraction. Removal of this contaminant from the enzyme fraction could not be achieved by DEAE-cellulose or phosphocellulose chromatography with Mg^{2+} present.

As implied in the above paragraph, removal of Mg^{2+} resulted in a decreased activity of the enzyme fraction, but it also resulted in separation of heme from the synthetase containing fraction. In the hope of establishing a compromise between reduction of enzyme activity and separation of heme (in a low Mg^{2+} environment), the use of EDTA on PC-I fraction was examined. Preliminary experiments indicated that the concentrated PC-I fraction of the enzyme was stable to EDTA when applied after the first phosphocellulose chromatography and subsequent enzyme concentration. This treatment, as described below, provided an efficient means of separating the contaminating heme without affedting the enzyme activity. From this stage on, the inclusion of 150 mM ammonium sulfate was necessary to maintain the stability of the enzyme and was therefore included whenever possible. Care was taken however, not to expose the enzyme to saturating concentrations of ammonium sulfate. Ammonium sulfate precipitation was found to cause aggregation of the enzyme, which introduced problems in the subsequent purification steps.

(e) EDTA Treatment and Subsequent Phosphocellulose Chromatography of PC-I fraction: PC-I fraction was dialyzed against Buffer TR2 containing 2 M sucrose and 150 mM ammonium sulfate for 10 hours. This

method of concentration allowed a 10-fold decrease in the volume of the fraction. To the concentrated PC-I fraction, EDTA (50 mM stock solution, pH 7.0) was added to a final concentration of 2 mM. The fraction was allowed to stand for 30 minutes on ice. The treated sample was then diluted with Buffer RS2 (see Chapter 2, Section II (v)), to reduce the ammonium sulfate concentration to 20 mM, and subsequently loaded onto a phosphocellulose column (1 cm x 40 cm) which had been equilibrated with Buffer RS2. The column was first washed with Buffer RS2 until the base line at 280 nm was obtained on the UV monitoring system (ISCO). After the base line was obtained the column was washed with the buffer containing 75 mM ammonium sulfate. The 75 mM ammonium sulfate fraction contained the heme formerly associated with the phenylalanyl-tRNA synthetase fraction. The synthetase fraction was eluted with a linear gradient of ammonium sulfate from 75 mM to 200 mM in 150 ml of Buffer RS2. Fractions of 1.6 ml were collected and those containing significant enzymic activity were pooled. The elution and activity profile obtained during this phosphocellulose chromatography is presented in Figure 6. It should be noted that although the synthetase fraction retained activity during this step, a prolonged exposure of the enzyme to low Mg^{2+} conditions resulted in a decrease in enzymic activity. Therefore, in order to stabilize the enzyme, the pooled fractions, designated as PC-II fraction was made 1 mM with respect to $MgCl_2$. This fraction was then concentrated by dialysis against Buffer TR2 containing 2 M sucrose and 150 mM ammonium sulfate.

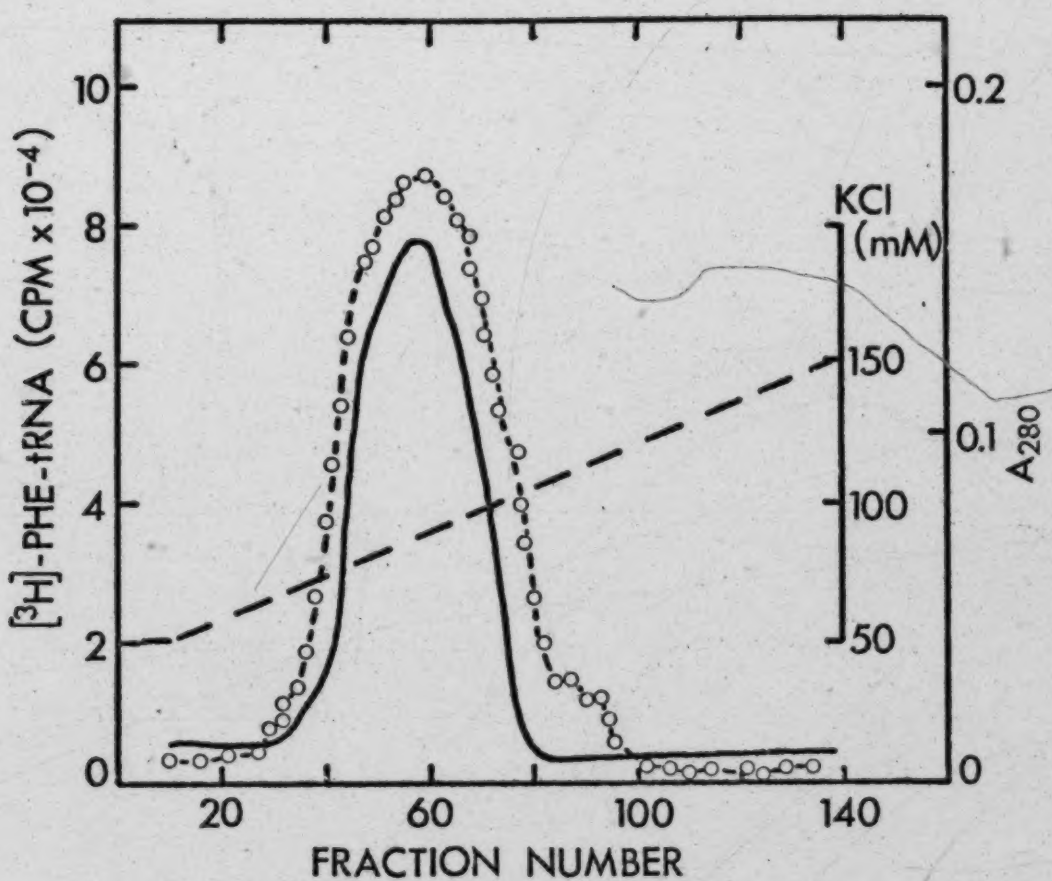


FIGURE 6: Elution profile of phe-tRNA synthetase from a phosphocellulose column chromatography. Concentrated PC-I fraction was applied to a phosphocellulose column (1 cm x 40 cm) and washed with Buffer RS2 containing 75 mM KCl. The synthetase was then eluted with a linear gradient of KCl from 75 to 200 mM. A_{280} recording on an ISCO UV-analyzer (—); phe-tRNA synthetase activity (0); KCl concentration (--). Fractions from 32 to 78 were pooled and condensed (PC-II fraction).

(f) Sephadex G-200 Column Chromatography of PC-II fraction: The condensed PC-II fraction was subjected to Sephadex G-200 column chromatography. This step permitted removal of residual impurities from the phenylalanyl-tRNA synthetase fraction and molecular weight estimation of the native enzyme. The column (150 ml bed volume) was thoroughly washed with Buffer TR2 containing 150 mM ammonium sulfate. The sample (1.2 ml) was loaded onto the column and eluted with the buffer. Figure 7 shows the elution profile obtained after chromatography of PC-II fraction on the Sephadex G-200 column. At this stage of purification, the major absorption peak coincided with the position of phenylalanyl-tRNA synthetase activity. A small amount of protein appeared at the low MW region of the elution. This minor component contained RNase, as determined by the method described in Chapter 2, Section III (xvi), and was well separated from the synthetase fraction. The fractions containing enzymic activity were pooled and dialyzed against Buffer TR2 containing 2.4 M sucrose and 150 mM ammonium sulfate. This condensed fraction, designated as SG-fraction, was stored at -20°C. At this stage, the extent of purification was approximately 2500-fold compared to AS-fraction (Table 5).

(ii) Physical Properties of Rabbit Liver Phenylalanyl-tRNA Synthetase

(a) Molecular Weight of Phenylalanyl-tRNA Synthetase by Molecular Sieve Chromatography: In order to obtain an approximate molecular weight for the purified phenylalanyl-tRNA synthetase (SG-fraction) in native form, a mixture of four marker proteins (thyroglobulin, glucose oxidase, ovalbumin, and cytochrome c) was applied to

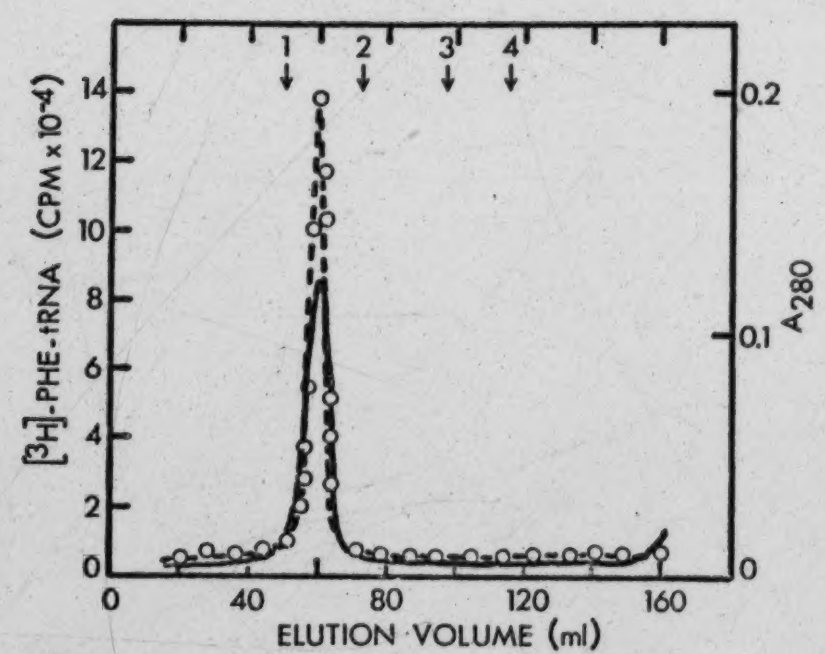


FIGURE 7: Elution profile of phe-tRNA synthetase on Sephadex G-200. The PC-I fraction (4 mg portions) was applied to a Sephadex G-200 column (2.5 cm x 45 cm) and eluted with Buffer TR2 containing 150 mM $(NH_4)_2SO_4$. Flow rate was maintained at 20 ml/hr. One-ml fractions were collected, and aliquots were assayed for synthetase activity (0). The 280 nm absorption was recorded on an ISCO UV analyzer (—). Immediately after the experiment, the mixture of four marker proteins was processed under identical conditions. The positions of these markers are indicated by arrows with numbers: 1. Thyroglobulin (MW = 6.6×10^5; 2. Glucose oxidase (MW = 1.86×10^5; 3. Ovalbumin (MW = 4.5×10^4; 4. Cytochrome c (MW = 1.24×10^4.

the column under identical conditions to those used for the synthetase (Chapter 2, Section III (xix)). The elution positions of the marker proteins from the column was then determined. In order to ensure that there was no aggregation between the marker proteins which could result in a faulty estimation of molecular weight, marker proteins were also processed individually on the Sephadex column. Both methods gave rise to the same elution position for each marker protein. The marker positions are indicated in Figure 7. According to these results, the approximate molecular weight of native phenylalanyl-tRNA synthetase was calculated to be 270,000.

(b) Subunit Structure of Phenylalanyl-tRNA Synthetase by Gel Electrophoresis: When our method for the purification of rabbit liver phenylalanyl-tRNA synthetase was developed, it was already apparent from other reports that synthetases were diverse with respect to subunit structure. Some were reported to be single polypeptide chains such as E. coli (184) and yeast (185) valyl-tRNA synthetase. Others were reported to consist of identical subunits such as the dimeric structure (α_2) of E. coli seryl-tRNA synthetase (21), and the tetrameric structure (α_4) of E. coli phenylalanyl-tRNA synthetase (20). Yet a third variety of synthetase structure was reported. This variety apparently consisted of subunits of differing molecular weights such as the tetrameric $\alpha_2\beta_2$ subunit structure of E. coli glycyl-tRNA synthetase (186), and yeast phenylalanyl-tRNA synthetase (181). Therefore, it was worthwhile to determine the subunit structure of rabbit liver phenylalanyl-tRNA synthetase.

The purified synthetase (SG-fraction) was subjected to SDS-gel electrophoresis at pH 7.1 as described in Chapter 2, Section III (xviii). Figure 8 illustrates a densitometric tracing of such a gel. The results indicated that purified phenylalanyl-tRNA synthetase, which migrated as a single band when subjected to acrylamide gel electrophoresis in the absence of denaturing agents, gave rise to two distinct components in the presence of SDS. Calculation of the molecular weight of the two components by comparison to the relative mobilities of several standard proteins (Chapter 2, Section III (xviii)) suggested a value of 76,000 for the larger subunit and 58,000 for the smaller subunit (Figure 9). Since the relative densities of the two subunits on the tracing were similar, two possible forms of the native enzyme were obvious; either phenylalanyl-tRNA synthetase was of the form $\alpha_1\beta_1$ (MW = 134,000) or $\alpha_n\beta_n$ (MW = multiple of 134,000). The fact that the molecular weight of the native enzyme, as determined by molecular sieving, was 270,000, indicates that phenylalanyl-tRNA synthetase from rabbit liver must be composed of four subunits of the form $\alpha_2\beta_2$.

(iii) Enzymic Properties of Rabbit Liver Phenylalanyl-tRNA Synthetase

(a) Specificity of Phenylalanyl-tRNA Synthetase: In order to examine the homogeneity of the phenylalanyl-tRNA synthetase in terms of the amino-acylation reaction, the specificity of the purified enzyme (SG-fraction) towards 16 labelled amino acids was examined as described in Chapter 2, Section III (xiii). As in the case of the amino acid acceptance studies describedin Chapter 3, both commercially available yeast tRNA and purified rabbit liver tRNA were used in the aminoacylation reaction. The

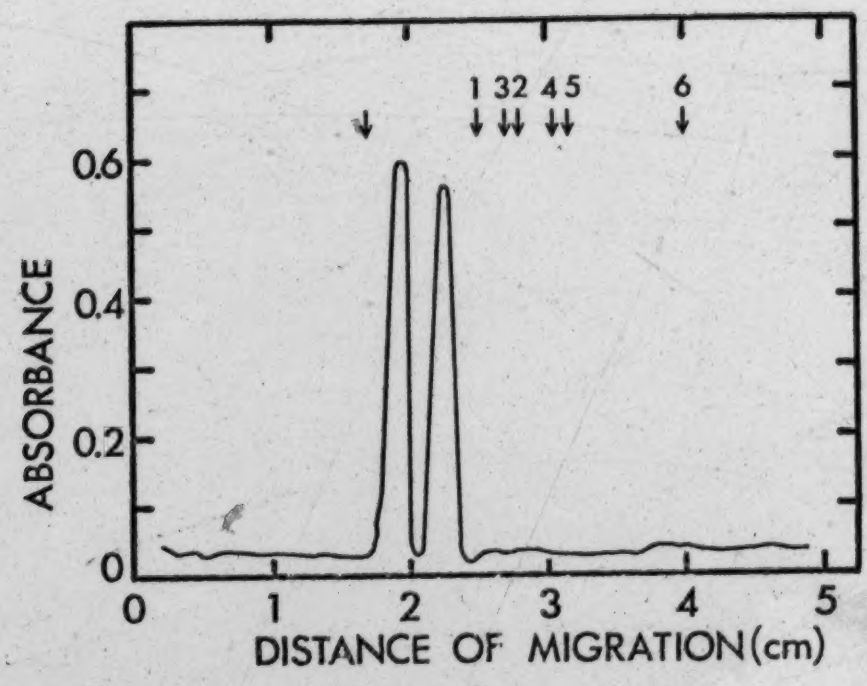


FIGURE 8: Densitometric tracing of SDS-gel after electrophoresis of phe-tRNA synthetase. The purified phe-tRNA synthetase (10 µg) was treated with SDS (2%) at 85°C for 15 min in the presence of 50 mM dithiothreitol. Then the sample was subjected to electrophoresis as described in Chapter 2, Section III (xviii). The gel was stained with Coomassie brilliant blue, and then destained. The densitometric tracing was made using an ISCO gel scanner Model 659. The relative positions of marker proteins which were treated exactly the same way are indicated by arrows with numbers: (1) Ovalbumin (MW = 4.5×10^4; (2) Aldolase subunits (MW = 4.0×10^4; (3) Succinyl-CoA synthetase β subunit (MW = 3.9×10^4; (4) Succinyl-CoA synthetase α subunit (MW = 2.85×10^4; (5) Chymotrypsin (MW = 2.75×10^4; (6) RNase A (MW = 1.37×10^4.

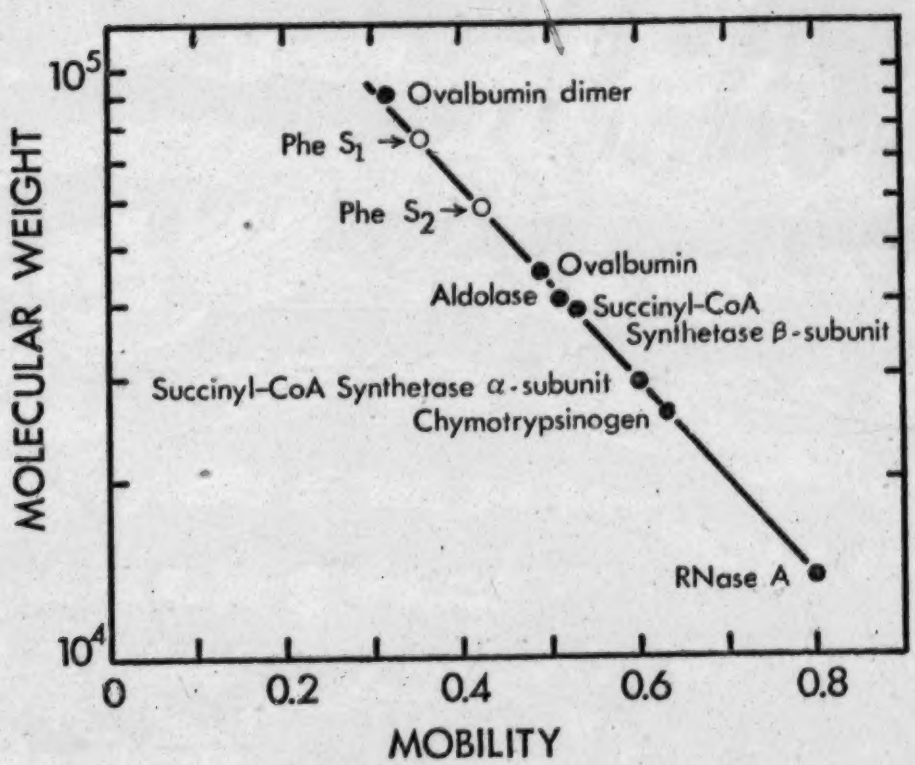


FIGURE 9: Molecular weight determination of the subunits of rabbit liver phenylalanyl-tRNA synthetase by SDS-gel electrophoresis as described in Chapter 2, Section III (xviii). The positions of phenylalanyl-tRNA synthetase are indicated by Phe S_1 (MW = 76,000 and Phe S_2 (MW = 58,000. The mobility of Succinyl-CoA synthetase was used as an internal standard.

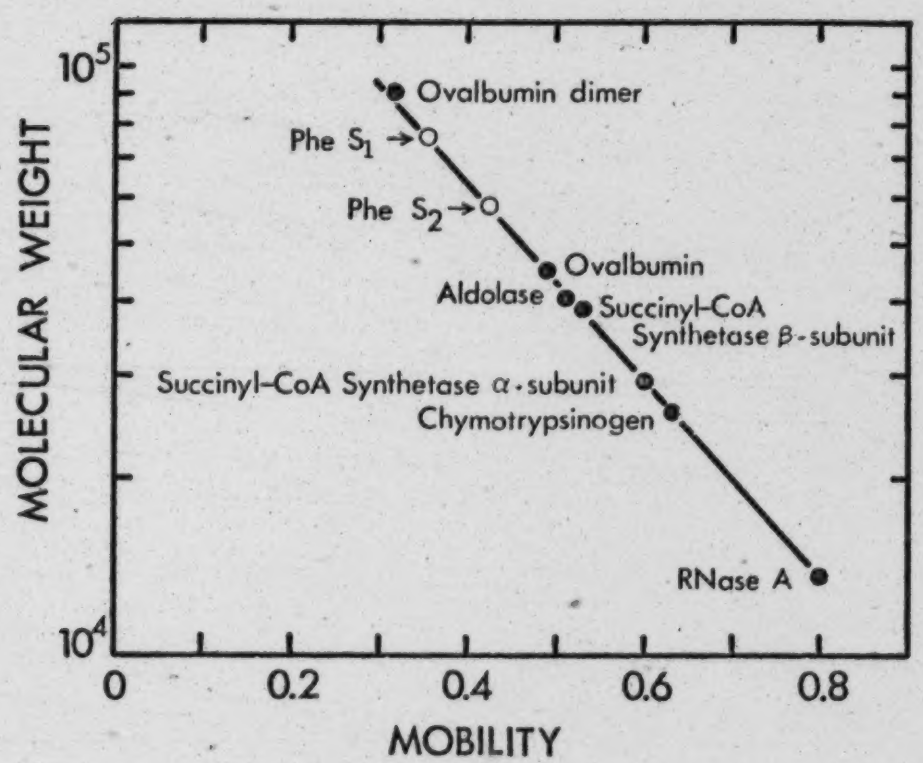


FIGURE 9: Molecular weight determination of the subunits of rabbit liver phenylalanyl-tRNA synthetase by SDS-gel electrophoresis as described in Chapter 2, Section III (xviii). The positions of phenylalanyl-tRNA synthetase are indicated by Phe S_1 (MW = 76,000 and Phe S_2 (MW = 58,000. The mobility of Succinyl-CoA synthetase was used as an internal standard.

results presented in Table 6 strongly suggest that the aminoacylation of both yeast and liver tRNA occurred only in the presence of phenylalanine. As expected from the results obtained in the amino acid acceptance studies (Chapter 3, Section II (iii)), aminoacylation of homologous rabbit liver tRNA was more efficient than for a stock of commercially available yeast tRNA. Thus, specificity of the enzyme towards phenylalanine was demonstrated.

(b) Optimum Reaction Conditions for Aminoacylation: As stated previously, the reason for preparing rabbit liver tRNA (Chapter 3) and rabbit liver phenylalanyl-tRNA synthetase was to obtain sufficient quantities of [^{3}H]-phenylalanyl-tRNA to examine the biological significance of a mRNA:ribosome complex. In order to make the preparation of [^{3}H]-phenylalanyl-tRNA as efficient as possible, it was necessary to establish optimum reaction conditions.

The optimum conditions for the aminoacylation reaction catalyzed by the purified phenylalanyl-tRNA synthetase were established by testing the effect of individual reaction components at various concentrations as described in the appropriate figure legends. The optimum pH for the aminoacylation reaction was between 7.8 and 8.0 (Figure 10A). The reaction was found to be totally dependent on the presence of Mg^{2+} with an optimum concentration of 20 mM (Figure 10B). KCl was not required for the reaction; in fact, concentrations higher than 40 mM inhibited the aminoacylation reaction (Figure 10C). Although the presence of β-mercaptoethanol was necessary for optimal aminoacylation, a critical concentration was not detected over the range examined.

TABLE 6

Amino Acid Specificity of Phenylalanyl-tRNA Synthetase

[^{14}C]amino acid	pmoles Incorporated	
	Yeast tRNA	Rabbit'Liver tRNA
Alanine	2.7	1.9
Arginine	4.8	5.9
Aspartic Acid	2.6	3.1
Glutamic Acid	2.3	2.0
Glycine	2.3	2.5
Histidine	6.2	8.6
Isoleucine	3.4	2.6
Leucine	3.5	3.1
Lysine	2.8	2.9
Methionine	2.7	2.8
Phenylalanine	161.7	303.6
Proline	3.9	5.4
Serine	3.4	3.8
Threonine	2.3	3.9
Tyrosine	15.2	25.5
Valine	2.9	3.5

Results are expressed as pmoles amino acid incorporated at 2 min of reaction. All [^{14}C] amino acids possess radioactivity = 50 mCi/mmole.

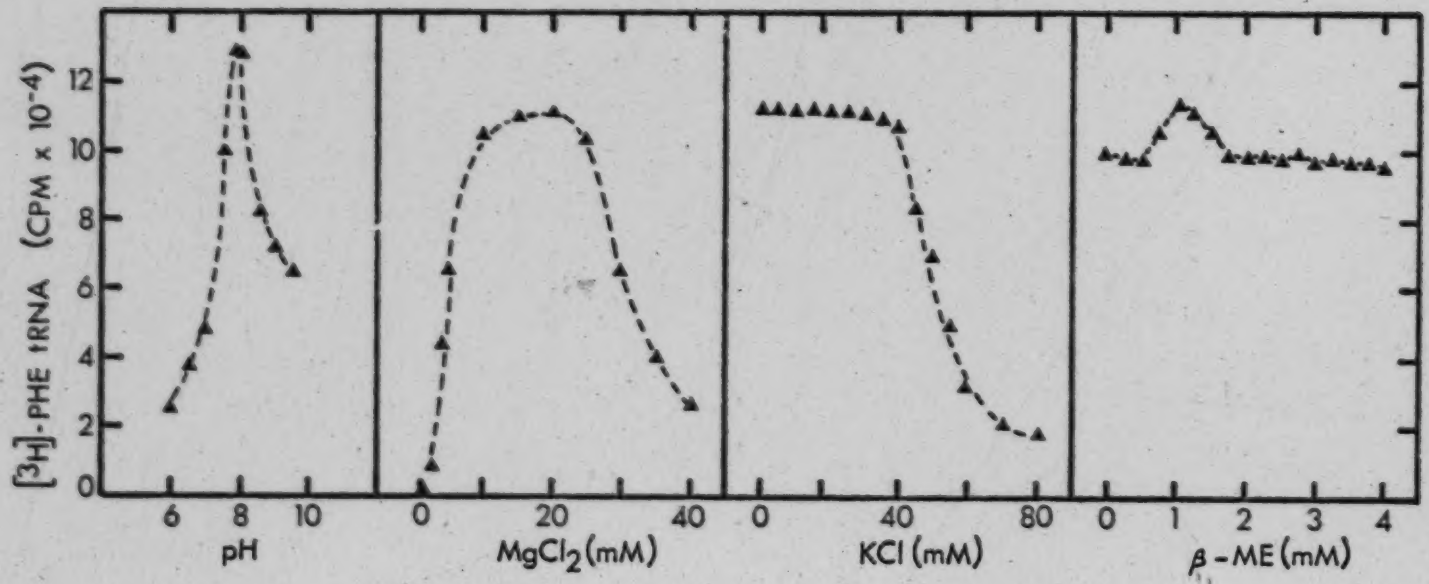


FIGURE 10: Optimum reaction conditions for rabbit phe-tRNA synthetase. The reaction was carried out as described in Chapter 2, Section III (xiii). (A) pH of the reaction mixture was set as indicated on the abscissa. The buffer used was Tris-HCl. (B) $MgCl_2$ concentration was modified as indicated on the abscissa. Other reaction components are at optimal concentrations. (C) KCl concentration was manipulated as indicated. (D) β-mercapto-ethanol concentration was manipulated as indicated.

(iv) Kinetic Properties of Phenylalanyl-tRNA Synthetase: At the time we initiated research into the properties of rabbit liver phenylalanyl-tRNA synthetase, little was known concerning the kinetics of the aminoacylation reaction in mammalian systems. Therefore, it was appropriate to examine the kinetic properties of phenylalanyl-tRNA synthetase, and the kinetic mechanism of the aminoacylation reaction catalyzed by the synthetase.

In the study of enzyme kinetics, the Michaelis constants K_m and V_{max} are two basic parameters; K_m is a measure of the affinity of an enzyme for a given substrate and V_{max} is a measure of the maximum velocity of the enzyme reaction. These parameters can be obtained by several methods. However, the most widely used is the Lineweaver-Burk plot (187). This method involves plotting the reciprocal of the initial velocity against the reciprocal of the substrate concentration. Details concerning the application of the Lineweaver-Burk plot are described by Cleland (188-191), and by Dixon (192). Apparent K_m^* and V_{max} values for each of the three substrates - tRNA, amino acid, and ATP- involved in the phenylalanyl-tRNA synthesizing reaction were obtained by the Lineweaver-Burk plot method.

The initial velocity was measured by the amount of [^{3}H]-phenylalanyl-tRNA synthesized at 2 min of reaction. The use of 2 minute measurements in the initial velocity experiments was justified as follows: the time course of [^{3}H]-phenylalanyl-tRNA synthesis as measured by the aminoacylation reaction was examined at different concentrations of synthetase. The results (Figure 11) indicated that for all concentrations of enzyme employed, the synthesis of [^{3}H]-phenylalanyl-tRNA

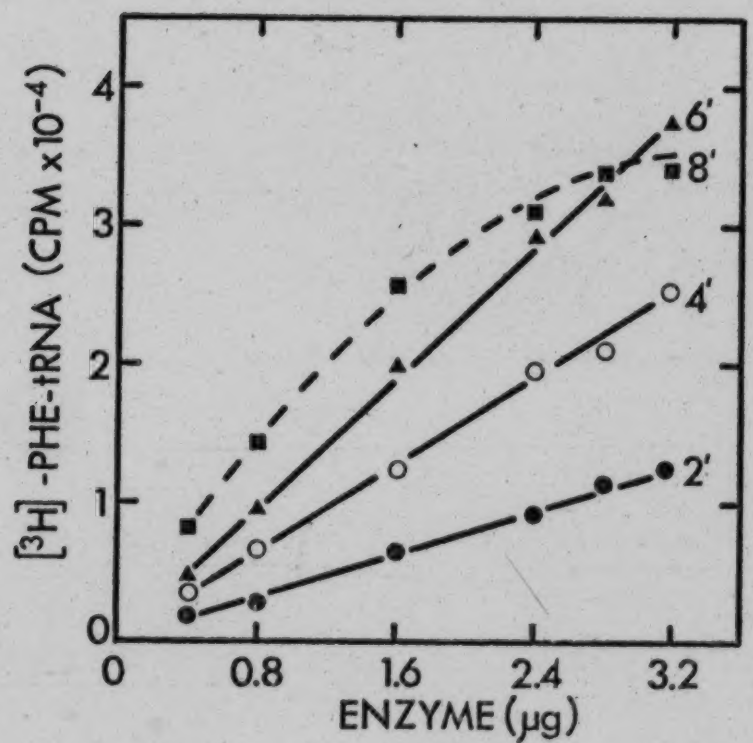


FIGURE 11: Time course of [^{3}H]-phenylalanyl-tRNA synthesis at different concentrations of phenylalanyl-tRNA synthetase. The experimental conditions in 0.5 mls are described in Chapter 2, Section III (xiii). For each concentration of enzyme examined (abscissa) 0.1 ml samples were withdrawn and processed (Chapter 2, Section III (i and iv) at time: 2 min (●), 4 min (○), 6 min (▲), and 8 min (■) after initiation of the reaction.

proceeded linearly for at least 6 minutes.

Average K_m and V_{max} values thus obtained from five sets of experiments are presented in Table 7. The order of affinity of the enzyme for each substrate was found to be: tRNA, phenylalanine, and ATP, in descending order. This relative order of K_m values is typical of that reported for other synthetases (193). It was also noted in these studies that ATP concentrations higher than 2 mM were inhibitory to the aminoacylation reaction. Similar observations have been reported for seryl- and valyl-tRNA synthetases from E. coli (194), and tryptophanyl-tRNA synthetase from human placenta (105).

It should be noted that at the time of the kinetic studies, purified $tRNA^{phe}$ was not available to us. For this reason we employed unfractionated tRNA as a substrate in the aminoacylation reaction. Final evaluation of the apparent kinetic parameters for the phenylalanyl-tRNA synthetase reaction requires $tRNA^{phe}$.

(a) Initial Velocity Studies: Cleland analysis of initial velocity patterns was used to gain insight into the kinetic mechanism of the phenylalanyl-tRNA synthetase reaction. In theory, for reactions involving more than one substrate, information can be obtained concerning the order of substrate binding to the enzyme. Such information can be obtained by examining the reciprocal plots of initial velocity versus substrate concentration at different fixed concentrations of a second substrate. According to Cleland analysis, two possible initial velocity patterns can occur: parallel lines and converging lines. Parallel lines are observed when no reversible connection exists between the points of

TABLE 7

Kinetic Parameters for Rabbit Liver phe-tRNA Synthetase

Substrate	K_m (M)	V_{max} (nmoles/min/mg)
L-phenylalanine	4.5×10^{-6}	98
ATP	3.2×10^{-5}	98
Yeast tRNA*	2.3×10^{-7}	91

* Corrected to approximate $tRNA^{phe}$ concentration.

combination of the variable and the fixed substrates; converging lines are observed when such a reversible connection exists. This method of analysis was applied to the phenylalanyl-tRNA synthetase-dependent aminoacylation reaction in order to examine the mode of binding of the three substrates - tRNA, phenylalanine, and ATP.

Results of the initial velocity studies for the phenylalanyl-tRNA synthetase reaction are presented in Figure 12. An intersecting pattern was obtained when 1/v was plotted against 1/[phe] at different fixed concentrations of ATP and at a saturating concentration of tRNA (Figure 12A). A linear converging pattern of reciprocal plots was also obtained when 1/v was plotted against 1/[phe] at different fixed concentrations of tRNA and at a saturating level of ATP (Figure 12B). Similarly, a linear converging pattern was obtained when the ATP concentration was varied at different fixed concentrations of phenylalanine (Figure 12C), and when ATP was varied at different fixed concentrations of tRNA (Figure 12D). Since only converging patterns were observed under all conditions where the concentrations of the three substrates was manipulated, all three substrates must be connected reversibly in the aminoacylation reaction sequence. According to Cleland analysis, the release of product would block the reversible connection in such a system described above. Therefore, these results can only be interpreted as evidence for a mechanism in which tRNA, phenylalanine, and ATP combine with the synthetase prior to the release of any product.

(b) Dead-end Inhibition Studies: Although examination of initial velocity patterns as described in section (a) provided useful information concerning the kinetic mechanism of the synthetase reaction, additional

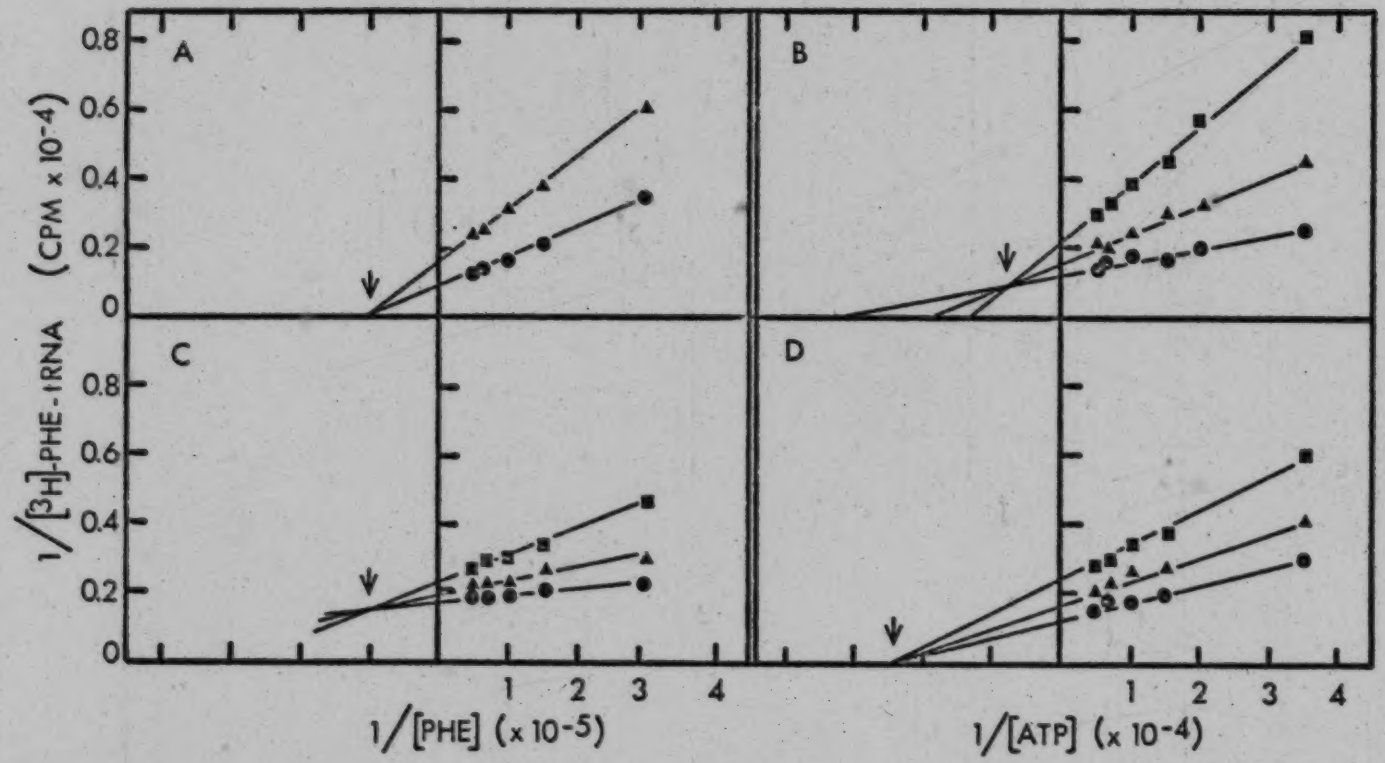


FIGURE 12: Initial velocity patterns for rabbit phe-tRNA synthetase. Plots were made for the reciprocals of the [^{3}H]-phenylalanine incorporated (expressed as CPM) at two min reaction versus the reciprocals of the molar concentrations of either L-phenylalanine (A and C) or ATP (B and D) at different fixed concentrations of either ATP (A), L-phenylalanine (B), or tRNA (C and D). Experimental conditions were described in Chapter 2, Section III (xiii). (A) tRNA 2 mM; ATP 0.5 mM (▲), 2 mM (●). (B) tRNA 2 mM; phenylalanine 3.3 μM (■), 10 μM (▲), 16.7 μM (●). (C) ATP 2 mM; tRNA 0.2 μM (■), 0.5 μM (▲), 1.5 μM (●). (D) Phenylalanine 13.3 μM; tRNA 0.2 μM (■), 0.5 μM (▲), 1.5 μM (●).

methods were desirable to verify the mode of substrate addition to the enzyme. Thus, the effect of dead-end inhibitors on the initial velocity patterns of the enzymic reaction was studied. Dead-end inhibitors have been defined by Cleland as compounds that are neither substrates nor products of the reaction but which react with certain enzymic forms to give complexes that are unable to form products. Such interactions result in three basic types of inhibition: (1) competitive inhibition, which results in a change in K_m but not V_{max}; (2) non-competitive inhibition, which results in a change in V_{max} but not in K_m; and, (3) uncompetitive inhibition, which results in a change in both K_m and in V_{max}. Competitive and non-competptive inhibition are characterized by linear converging patterns on the Lineweaver-Burk plot; uncompetitive inhibition is characterized by parallel patterns. Depending on the choice of dead-end inhibitors, the type of pattern obtained can provide additional information concerning the mechanism of the phenylalanyl-tRNA synthetase reaction.

Two dead-end inhibitors were selected: L-phenylalanine amide, an analogue of phenylalanine, and β,γ-methylene ATP, an analogue of ATP. The effects of these dead-end inhibitors on initial velocity patterns for the aminoacylation reaction are presented in Figure 13. When ATP concentration was varied at different fixed concentrations of methylene ATP, a competitive inhibition pattern was obtained (Figure 13A). According to Cleland analysis, this suggests that both ATP and methylene ATP combine with the same enzyme form. In contrast to this, methylene ATP was found to produce a non-competitive pattern for both phenyl-

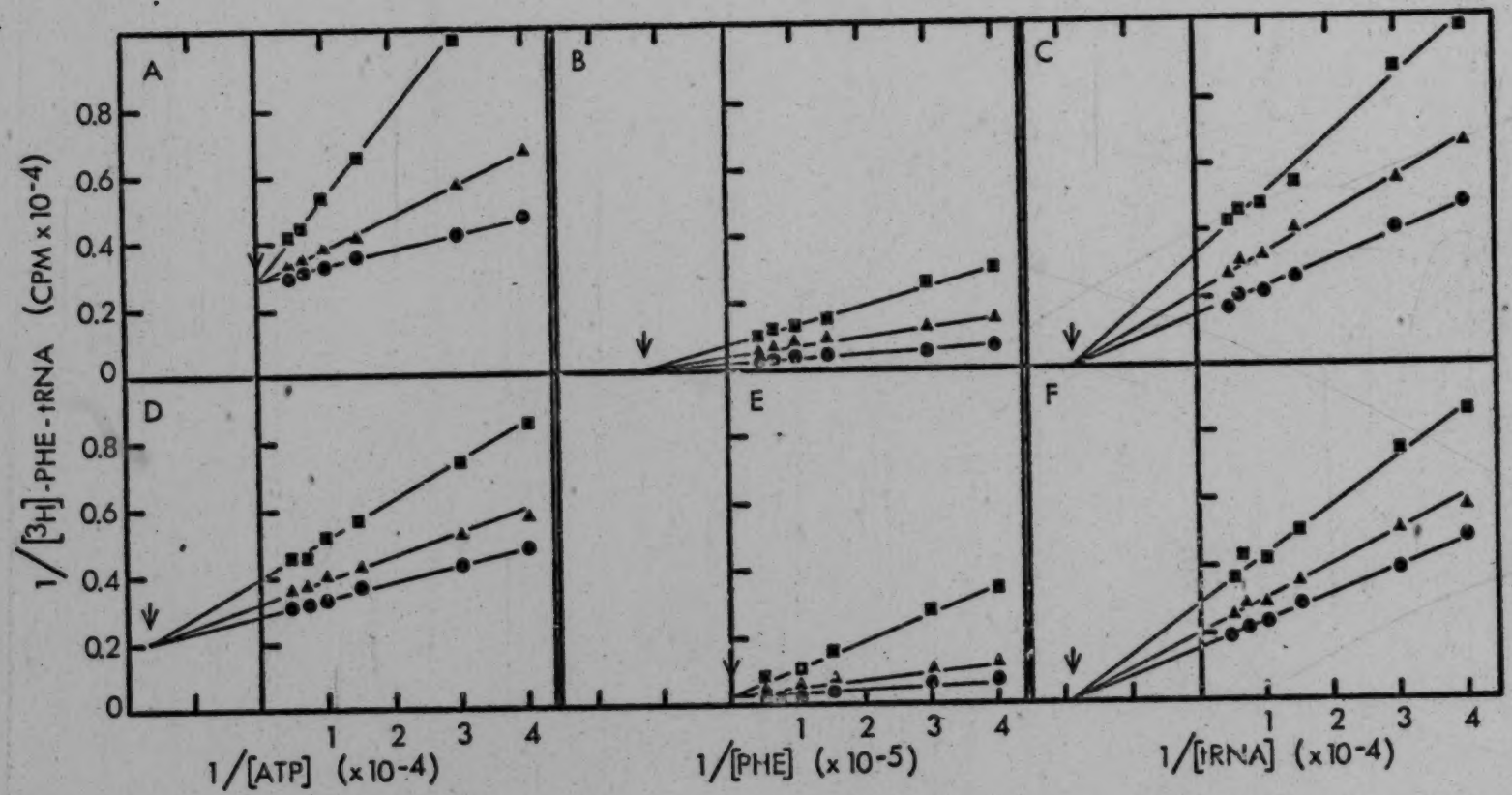


FIGURE 13: Inhibition of the phe-tRNA synthetase reaction by β,γ-methylene ATP and L-phenylalanine amide. Plots were made for the reciprocals of initial velocity versus the reciprocals of the molar concentrations of ATP (A and D), L-phenylalanine (B and E), and tRNA (C and F). The concentration of β,γ-methylene ATP (A, B, C) were: 0 M (●), 0.6 x 10^{-4} M (▲), and 1.8 x 10^{-4} M (■). The concentrations of L-phenylalanine amide (D, E, F) were: 0 M (●), 2.4 x 10^{-5} M (▲), and 2.4 x 10^{-4} M (■). Experimental conditions were described in Chapter 2, Section III (xiii). Concentrations of the non-variable substrates were: ATP 1 mM, phenylalanine 10 μM, and tRNA 2 mM.

alanine and tRNA (Figure 13B and 13C). This result suggests that the inhibitor is interacting with a different enzyme form than phenylalanine and tRNA are. Moreover, the fact that the plots were converging rather than parallel suggested that the different enzyme forms are connected reversibly within the aminoacylation reaction sequence.

When phenylalanine concentration was varied at different fixed concentrations of phenylalanine amide, competitive inhibition patterns were observed (Figure 13E), whereas non-competitive patterns were obtained with ATP and tRNA (Figure 13D and 13F). These results suggest that both phenylalanine amide and phenylalanine combine with a single enzyme form which is different from those for ATP and tRNA. However, the converging patterns indicate that the enzyme forms are connected reversibly in the reaction sequence.

Since dead-end inhibitors interrupt the reversibility of the reaction sequence at the point of inhibitor binding, non-competitive patterns will be observed only if the point of enzyme-substrate interaction follows the point of addition of the inhibitor in the reaction sequence and is reversibly connected to it. A parallel pattern representative of uncompetitive inhibition would be expected if the binding of the variable substrate to the enzyme preceeded the point of addition of the inhibitor in the reaction sequence. In fact, the results obtained with methylene ATP and phenylalanine amide demonstrate competitive inhibition patterns when the variable substrates were ATP and phenylalanine, respectively, and non-competitive inhibition patterns for the remaining substrates.

Therefore, the results obtained with methylene ATP and phenylalanine amide imply that both ATP and phenylalanine bind first to the same enzyme form in a reaction sequence where the addition of the substrates is reversibly connected. The only mechanism which can accommodate an enzyme form which will bind either ATP or phenylalanine is one involving a random order of addition of all reactants to the enzyme prior to the release of any product. The results do not support the involvement of a Ping-pong mechanism - binding of substrates to the enzyme interrupted by release of product. Other studies of reactions involving three substrates and three products provided evidence for a random mechanism. For example, plots of the initial velocity of the formyl-tetrahydrofolate synthetase reaction (195,196), and more recently the E. coli arginyl-tRNA synthetase reaction (197) gave intersecting Line-weaver-Burk plots consistent with a random mechanism.

It is obvious that further studies must be conducted before definite conclusions can be made concerning the mechanism of the phenylalanyl-tRNA synthetase reaction. Foremost in these studies are steady state kinetic analyses. This method involves the analysis of exchange rates that occur when a trace amount of substrate or product, labelled with radioisotopes of high specific activity, is added to an enzyme reaction after it has reached equilibrium. The most common measurement for synthetase reactions involves ATP-$^{32}PP_i$ exchange. Recently ATP-$^{32}PP_i$ measurements were used to demonstrate a random order of substrate binding to E. coli methionyl-tRNA synthetase (198).

(c) Inhibition by Aurintricarboxylic Acid (ATA): ATA, a triphenyl-methane dye, is considered to be a specific inhibitor of the initiation

process of protein synthesis in the E. coli system (182). As will be demonstrated in Chapter 6, ATA also exhibits a high affinity for mRNA binding proteins (M-factor) and thereby prevents mRNA binding to the protein factors. In addition, studies carried out in this laboratory with E. coli tRNA 3'-terminal nucleotidyl transferase (199), DNA-dependent RNA polymerase (200), and R17 RNA dependent RNA polymerase (201), suggest that the inhibitory action of ATA is not confined to the binding of mRNA to ribosomes during initiation of protein synthesis. In fact, these studies provide strong evidence that ATA inhibits many RNA-protein interactions. We decided therefore to examine the effect of ATA on the phenylalanyl-tRNA synthetase-dependent aminoacylation reaction.

The results of these studies (Figure 14) indicate that ATA did inhibit the aminoacylation reaction. Based on the estimated molecular weight of phenylalanyl-tRNA synthetase, the upper limit of the molar ratio of ATA to enzyme was calculated to be 5-10 at the 50% inhibition concentration (I_{50}). Inhibition could be relieved by increasing the enzyme concentration Moreover, varying the concentration of the three substrates in the reaction did not alter the degree of ATA inhibition. Taken together, these facts suggest that the site of ATA interaction was on the enzyme. In this regard, it is noteworthy that naphthalene sulfonate, a known fluorescence probe, binds to the synthetase but does not inhibit its action (Figure 14).

Further support for a specific interaction between ATA and phenylalanyl-tRNA synthetase was obtained from experiments in which the order of adding reaction components was varied. The results (Table 8)

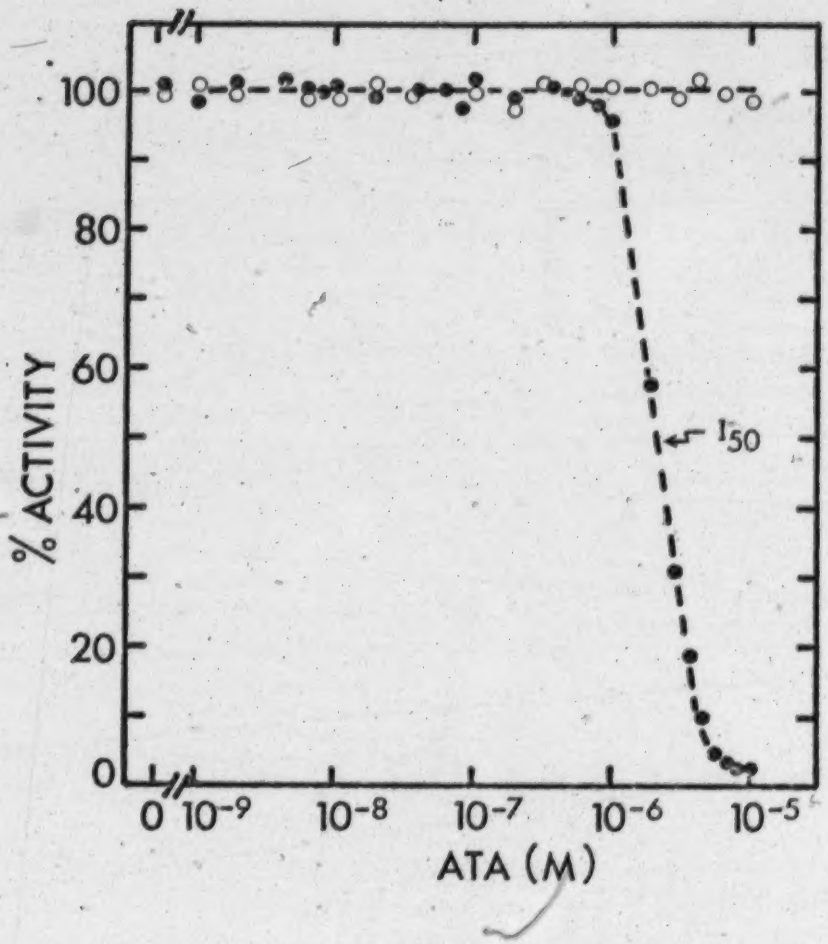


FIGURE 14: Effect of aurintricarboxylic acid and napthalene sulfonate on phe-tRNA synthesis. [^{3}H]phe-tRNA synthesis was measured as described in Chapter 2, Section III (xiii), except that ATA (●) or napthalene sulfonate (0), was added to the reaction mixture to a final concentration as indicated on the abscissa. All reaction components except the enzyme were mixed, and the reaction was initiated by adding the enzyme. The results were expressed as percent radioactivity relative to that in the control set which did not contain ATA. One hundred percent equivalent to 20,000 c.p.m. at 2 min reaction.

TABLE 8

ATA Inhibition Modified by Different Sequential Additions of Reaction Components

Experimental Set	Order of Addition	% [^{3}H]phe-tRNA Synthesized
1	Enzyme:(ATP,phe,tRNA)	100.0
2	Enzyme:ATA:(ATP,phe,tRNA)	9.5
3	Enzyme:ATP:ATA:(phe,tRNA)	8.7
4	Enzyme:[^{3}H]phe:ATA:(ATP,tRNA)	7.7
5	Enzyme:tRNA:ATA:(ATP,phe)	45.3
6	Enzyme:(ATP,phe):ATA:tRNA	9.4
7	Enzyme:(ATP,tRNA):ATA:phe	43.8
8	Enzyme:(phe,tRNA):ATA:ATP	40.1
9	Enzyme:(ATP,phe,tRNA):ATA	81.5

Components in parentheses are mixed prior to addition in the sequence. Additions were made at 5 minute intervals.

indicate that, when ATA was added to the enzyme prior to substrate addition, essentially 100% inhibition was obtained (set 2). On the other hand, when ATA was added to the enzyme-substrate mixture, its inhibitory effect was reduced (set 9). The effect on the ATA inhibition of adding substrates singly or in various combinations was also examined (sets 3-9). The most significant result obtained was that exposure of the synthetase to tRNA prior to the addition of ATA greatly reduced the inhibitory effect of the dye. However, addition of tRNA to an ATA-enzyme mixture had no effect on ATA inhibition of the aminoacylation reaction. Unlike tRNA, phenylalanine and ATP had no protective effect on the synthetase. This data not only supports the possibility that ATA is interacting with the phenylalanyl-tRNA synthetase but also suggests that the interaction might involve tRNA binding to the enzyme.

In order to examine the mode of ATA inhibition, initial velocity studies were carried out in the presence of varying concentrations of ATA. The data were examined on Lineweaver-Burk plots as presented in Figure 15. In these experiments, as with those conducted in obtaining the kinetic parameters (section (iv)), two substrates were held at fixed, near-saturating concentrations while the concentration of the third substrate was varied. A simple-linear, non-competitive pattern was obtained for all three substrates. This result suggests that, although the aminoacylation reaction appears to be inhibited by the interaction of ATA with phenylalanyl-tRNA synthetase, the interaction does not involve the substrate binding sites. The mechanism by which tRNA protects the enzyme against ATA remains a matter of speculation.

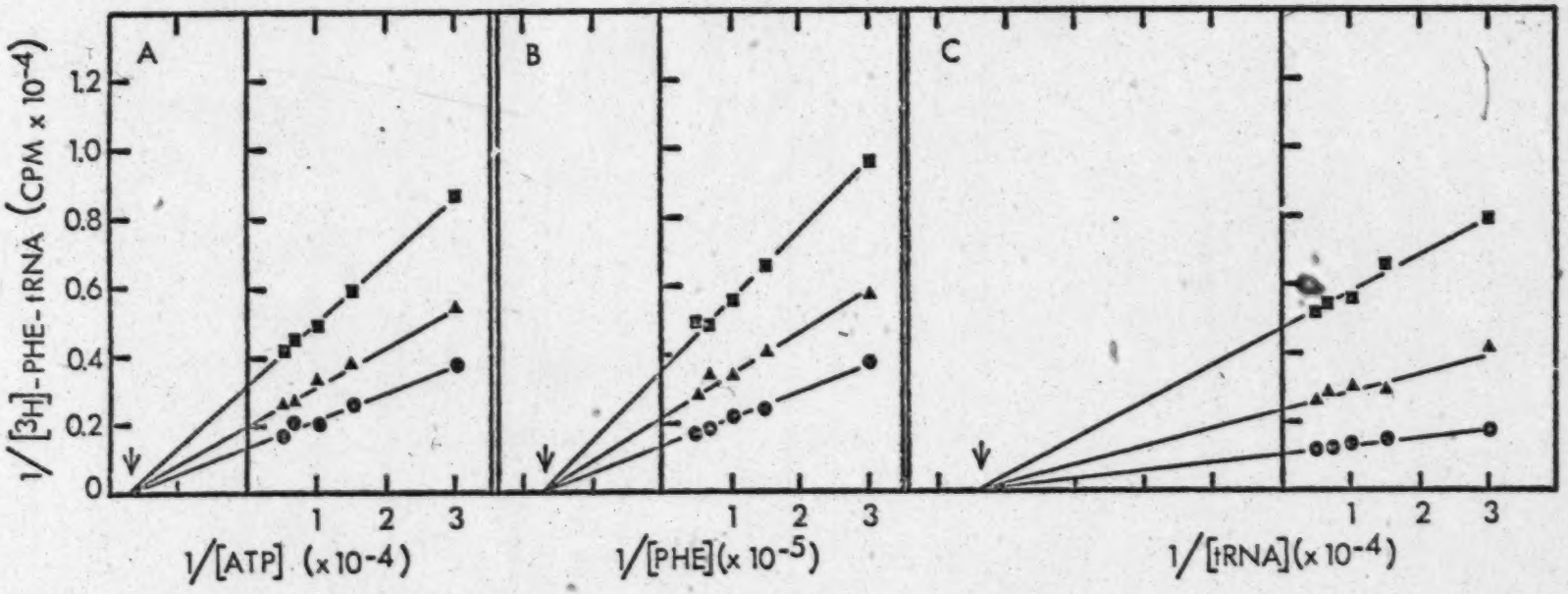


FIGURE 15: Inhibition pattern of phe-tRNA synthetase by ATA. Plots were made for the reciprocals of initial velocity versus the reciprocals of the molar concentration of ATP (A), L-phenylalanine (B), and tRNA (C) in the presence of various concentrations of ATA. no ATA (●); 1.5×10^{-6} M ATA (▲); 2.5×10^{-6} M ATA (■). Experimental conditions were described in Chapter 2, Section III (xiii). Concentrations of the non-variable substrates were the same as in Fig. 13.

In order to establish a reference system to ensure the validity of the ATA kinetic studies, the action of an inhibitor with a known effect on the aminoacylation reaction was examined. The inhibitor chosen was AMP, which is a product inhibitor in the aminoacylation reaction and as such was expected to demonstrate competitive inhibition with respect to ATP. The effect of AMP on the initial velocity of the aminoacylation reaction is presented in Figure 16. When 1/v was plotted against 1/[ATP] for experiments conducted at saturating levels of tRNA and different fixed levels of AMP, a competitive inhibition pattern was obtained (Figure 16A). Non-competitive patterns were obtained when either phenylalanine or tRNA concentrations were varied at different fixed concentrations of AMP (Figure 16B and 16C). These results, as expected, indicated that AMP competed for the ATP binding site on the synthetase, but not for the phenylalanine or the tRNA binding sites. These product inhibition studies support the interpretation of our ATA kinetic studies. Therefore the results obtained with regard to ATA inhibition lead us to conclude that the dye binds with phenylalanyl-tRNA synthetase at site(s) other than the substrate binding sites. It is conceivable that the inhibition of enzymic activity by ATA binding is due to an alteration of enzyme configuration upon dye binding.

III. Discussion

The purification to homogeneity of phenylalanyl-tRNA synthetase from rabbit liver was achieved as judged by the specificity towards the substrate and by the formation of only two bands upon SDS-gel electrophoresis. The molecular weights of the two bands correspond to 58,000

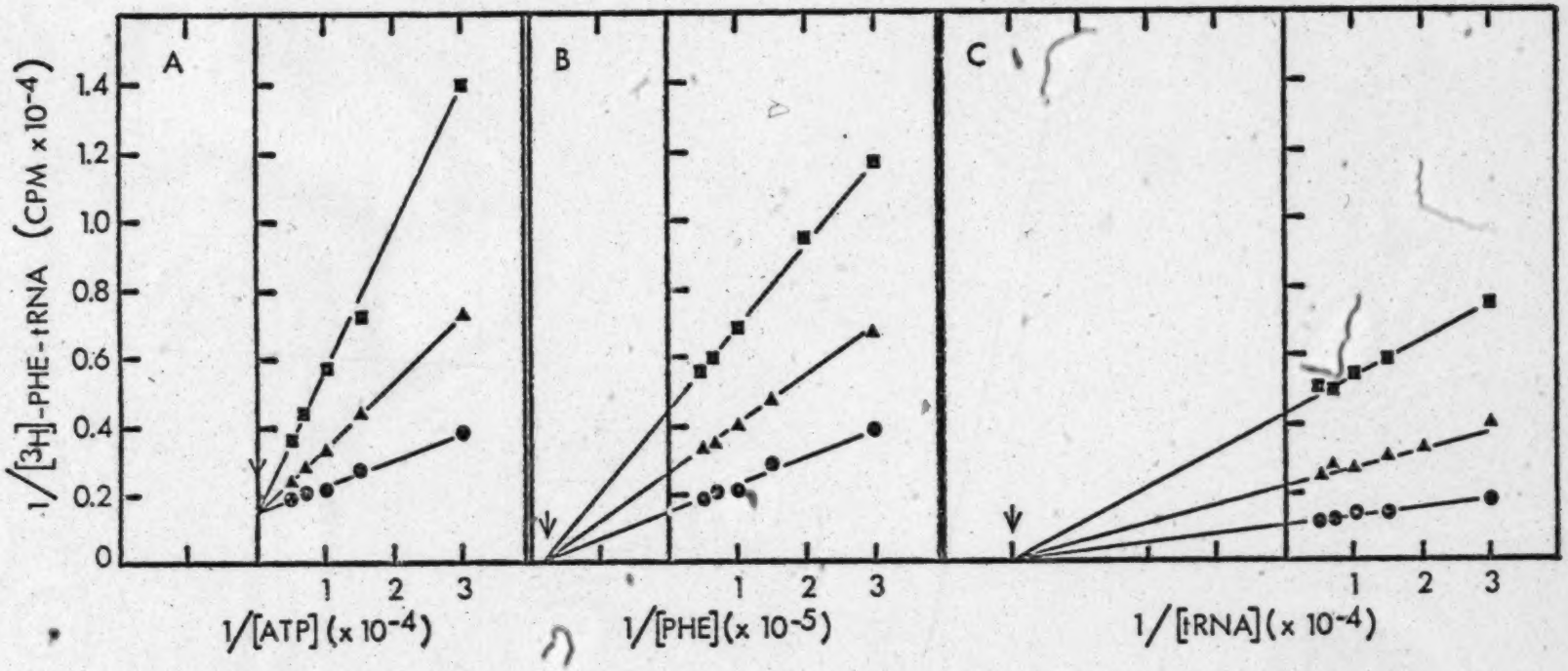


FIGURE 16: Inhibition of the phe-tRNA synthetase reaction by AMP. Plots were made for the reciprocals of initial velocity versus the reciprocals of the molar concentrations of ATP (A), L-phenylalanine (B), and tRNA (C). AMP concentrations were: 0 (●), 0.5 mM (▲), and 1 mM (■). Experimental conditions were described in Chapter 2, Section III (xiii). Concentrations of non-variable substrates were the same as in Fig. 13.

and 76,000. The molecular weight of the native enzyme by gel filtration was found to be 270,000. Based on these data, phenylalanyl-tRNA synthetase has a subunit structure of the form $\alpha_2\beta_2$ (α = 58,000, β = 76,000). Thus, all three known phenylalanyl-tRNA synthetases from different organisms (E. coli, yeast, and mammalian) possess a similar tetrameric structure (202, 181, 126). However, the molecular weight of the subunits differs among the phenylalanyl-tRNA synthetases from E. coli (α = 37,000, β = 98,000) (202) as compared to those from yeast (α = 63,000, β = 75,000) (181), and rabbit liver (α =58,000, β = 76,000 (126).

Although the results obtained in the substrate specificity studies indicate that both yeast and rabbit liver tRNA accept phenylalanine, there is a different in the efficiency of phenylalanine acceptance between heterologous yeast tRNA and homologous rabbit liver tRNA when the purified enzyme is used. Rabbit liver tRNA showed twice the amino acid acceptance activity relative to yeast tRNA. This phenomenon is consistent with the results obtained in the amino acid acceptance studies described in Chapter 3 using a crude synthetase fraction. Similar results were reported for the rat liver system (167, 102).

The apparent Michaelis constants determined for the three substrates in this chapter are similar to the values reported for other synthetases (102,105,203). However, small variations in K_m values for individual synthetases are present, particularly with respect to tRNA. This deviation of approximately a factor of 10 may be caused by our use of unfractionated tRNA instead of the purified $tRNA^{phe}$ used in other studies (102). The purification of $tRNA^{phe}$ from rabbit liver is required to obtain precise

values.

The kinetic analysis developed by Cleland has proven useful in discriminating among alternative reaction mechanisms. With this approach three types of kinetic mechanisms have been reported for aminoacyl-tRNA synthetases (104,105). In the first class, the release of the reaction products is ordered, as in the case of E. coli tryptophanyl- and prolyl-tRNA synthetases (204). In the second class, the product release is random, as is the case of human tryptophanyl-tRNA synthetase (105). Both these classes possess a ping-pong mechanism with an ordered addition of substrates to the enzyme. The third class is different from the above two classes in that the substrate addition is random as is the case of E. coli tyrosyl-tRNA synthetase (205), and E. coli arginyl-tRNA synthetase (197). Results presented in this chapter indicate that rabbit liver phenylalanyl-tRNA synthetase belongs to the third class. This prediction was based on two types of experiments. The first was the examination of reciprocal plots of initial velocity versus concentration of one of the substrates at different fixed concentrations of a second substrate. The second was the examination of the effect of the dead-end inhibitors, phenylalanine amide and β,γ-methylene ATP, on the initial velocity patterns for the aminoacylation reaction. These results favor a mechanism in which all three substrates, ATP, phenylalanine and tRNA interact with the synthetase in a random order. Moreover, the results suggest that all substrates must bind with the enzyme before any product is released. However, steady state isotope exchange studies have not been carried out to confirm these results and to determine the order of product release.

The effect of ATA on purified phenylalanyl-tRNA synthetase was of considerable interest. As stated previously, ATA was reported to be a specific inhibitor of mRNA binding during the initiation process of protein synthesis in E. coli (182). Recent studies in another laboratory (206), as well as our own (199-201), indicate that ATA inhibits most enzymic reactions involving RNA as template and primer. The inhibition of the phenylalanyl-tRNA synthetase-catalyzed aminoacylation reaction by ATA represents a further example of this concept. It is also noteworthy that ATA is not a competitive inhibitor for any of the three substrates in this synthetase system, rather it is a noncompetitive inhibitor. Once the enzyme substrate complex is formed however, ATA is no longer inhibitory. Therefore, it seems probable that ATA binds to the enzyme at a site(s) other than the substrate binding sites. This binding may cause conformational changes in the enzyme such that substrate binding is prevented and/or transfer of the amino acid to the tRNA is prevented. Fortunately, ATA is a fluorescent compound, therefore ATA-enzyme interaction is amenable to fluorescent probe studies. In this regard it was surprising to find that another fluorescent compound, napthaline sulfonate, can bind to the enzyme but does not inhibit the aminoacylation reaction.

With the purification and characterization of rabbit liver phenylalanyl-tRNA synthetase the second requirement toward establishment of an homologous cell-free system in which to examine mRNA:ribosome complex formation was accomplished.

CHAPTER 5

LARGE SCALE PREPARATION OF HOMOGENEOUS 80S RIBOSOMES FROM RABBIT LIVER

I. Introduction

Chapters 3 and 4 described the preparation of homologous tRNA and phenylalanyl-tRNA synthetase from rabbit liver. These components were required for the preparation of [^{3}H]-phenylalanyl-tRNA. The labelled aminoacyl-tRNA thus prepared provided us with the necessary RNA binding species (aminoacyl-tRNA and synthetic mRNA) to conduct our mRNA:ribosome complex formation studies (see Figure 1). However, before these components could be used, one obvious task remained - the preparation of ribosomes. This chapter describes the preparation of homogeneous 80S ribosomes from rabbit liver.

Although the involvement of ribosomes in protein synthesis was first explored in the mammalian system (207,208) and demonstrated in a mammalian cell-free system (3), the major contributions to our knowledge of the molecular events taking place during the process of polypeptide synthesis were made in the bacterial system (Chapter 1). Progress in mammalian ribosome systems began only toward the end of the 1960's. This delay in progress has been attributed to several factors: attempts to obtain pure homogeneous ribosomes from mammalian sources often resulted in low yield and loss of biological activity. Moreover, the quality of ribosomes prepared from various tissues was not consistent, as indicated by the variable ratios of RNA to protein in the

ribosomal particles (209,210). In general, mammalian ribosome preparations contained more proteins than expected for prokaryotic ribosomes (211). This feature suggests that (1) there is heterogeneity among ribosomal populations prepared from different tissues by different methods, and (2) there is contamination by non-ribosomal proteins. It has been reported, for example, that contamination of the ribosomal preparation with aminoacyl-tRNA:protein transferase makes the analysis of cell-free protein synthesis very difficult (212).

This chapter describes a method for the preparation of rabbit liver ribosomes in large quantities; at least 10,000 A_{260} units of biologically active 80S ribosomes could be prepared at one time. The ribosomes thus prepared were found to be free of RNase, aminoacyl-tRNA synthetase, and aminoacyl-tRNA: protein transferase activities. The procedure is not new in principle, but involves appropriate manipulation by: (1) deoxycholate treatment of tissue homogenate, a procedure which frees ribosomes from membranous structures and also reduces the Mg^{2+} concentration in the solution to avoid ribosome aggregation during the subsequent purification procedure (213), (2) polyethylene glycol-dextran sulfate partition of the ribosomal fraction, a procedure which reduces the volume of the ribosomal fraction to one-tenth of the original liver homogenate, (3) DEAE-cellulose chromatography of the ribosome fraction, a procedure which purifies the ribosomes by permitting complete removal of RNase and other non-ribosomal proteins from the ribosomes.

With this procedure, a kilogram quantity of rabbit liver could be processed within 48 hours. Ribosomes thus prepared were found to possess a constant RNA:protein ratio (0.95) and were active in poly-

uridylate-directed phenylalanine incorporation in the presence of added protein factors.

II. Results

(i) Preparation of Rabbit Liver Ribosomes

(a) Extraction of Rabbit Liver Tissue: For convenience, the isolation procedure to be described is with respect to 100 grams of rabbit liver. Actual preparations involved from 500 to 1000 grams of liver, depending on the availability of fresh rabbit liver. It should be pointed out that in order to minimize degradation of the tissue, the isolation procedure was carried out immediately after the liver had been excised from the rabbit and all subsequent procedures were carried out in the cold (0-4°) unless otherwise specified.

Liver from freshly bled rabbits was mined and homogenized in 2 volumes (w/v) of Buffer L (see Chapter 2, Section II (i)). Homogenization was carried out in a one-quart stainless steel Waring Blender (Model 5011) operated at high speed for 30 seconds. Deoxycholate was then added to the homogenate to a final concentration of 10 mM. This treatment was previously reported to liberate membrane bound ribosomes by disrupting the membrane structures (213). In order to degrade the DNA liberated from nuclei, the homogenate was treated with DNase at a final concentration of 5 µg/ml.

In order to ensure complete disruption of the tissue, the volume of the homogenate was increased to 450 ml with Buffer L and homogenized with two strokes of an electrically driven, tight-fitting, teflon homogenizer operated at medium speed. Cellular debris was removed by

centrifugation at 12,000 x G for 30 minutes in a Beckman J21 centrifuge.

(b) Polyethylene Glycol-Dextran Sulfate Parition: In preparation for the partition procedure, solid sodium chloride was added slowly to the above supernatant with stirring, to a final concentration of 350 mM, thus making the overall concentration of monovalent cation 400 mM. This concentration was found to be critical for the subsequent partition step. Moreover, addition of potassium chloride in the place of sodium chloride did not permit an effective partition. The reason for this preference for sodium is not known. Once the sodium chloride was completely dissolved, solid polyethylene glycol was added slowly to give a final concentration of 9.5%. The mixture was stirred for 15 minutes and then solid dextran sulfate was slowly added to a final concentration of 2.2%. Immediately a brown precipitate was observed; this was routinely taken as an indication of successful partition. The mixture was stirred for an additional 30 minutes and then allowed to stand in the cold for 60 minutes. During this period, the partition was completed. The top layer was removed by aspiration and discarded. The bottom dextran layer, containing the ribosomes, was concentrated by centrifugation at 8,000 x G for 5 minutes. The resulting dextran layer represented one-tenth of the original partition mixture.

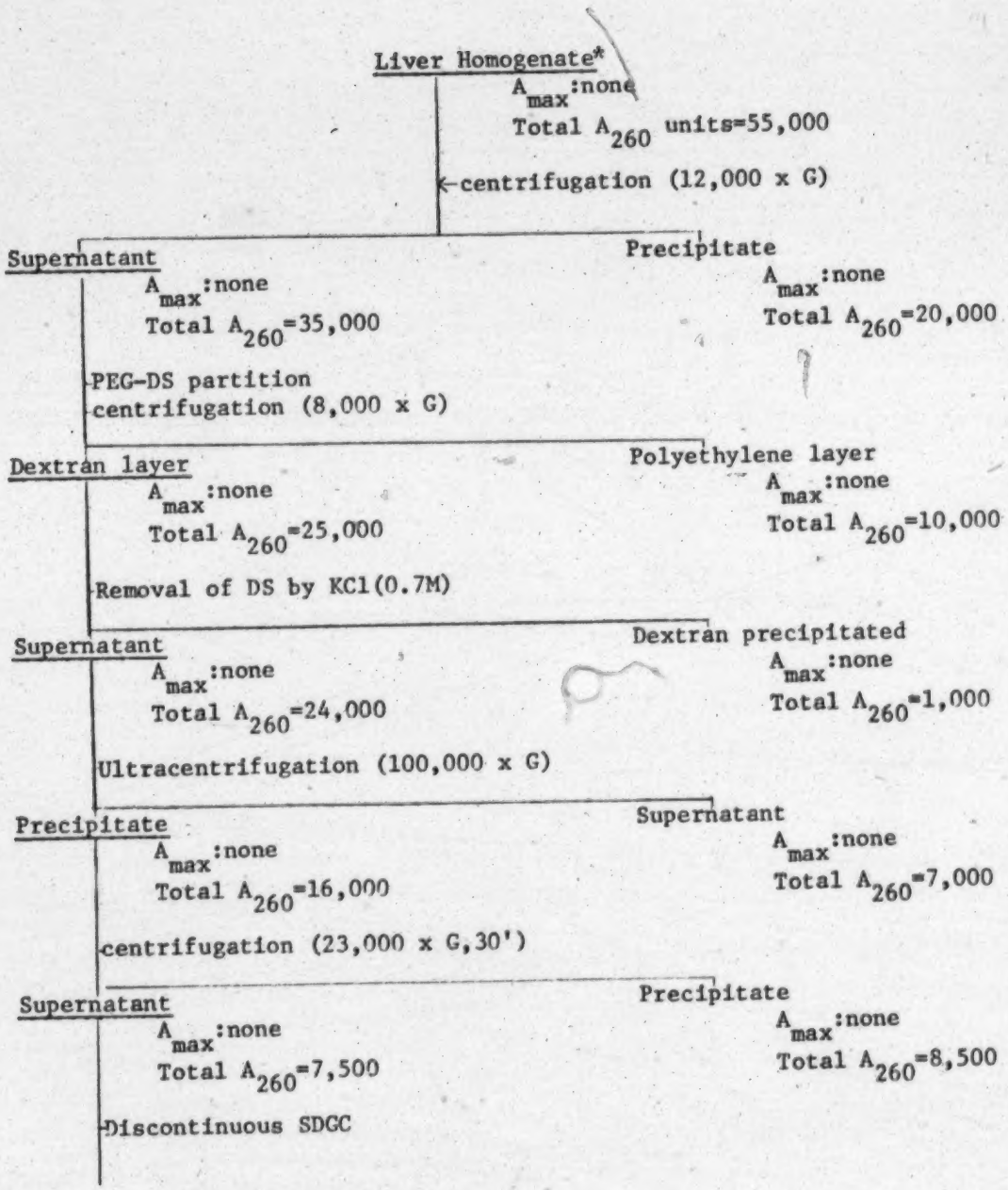
At this stage it was necessary to remove the dextran sulfate from the ribosomal fraction in order to permit subsequent ultracentrifugation. The removal of dextran sulfate was achieved by precipitation at high ionic strength (214). Solid potassium chloride was added to the fraction with stirring to give a final monovalent cation concentration of 0.7 M.

As soon as the salt was added the dextran sulfate precipitated out. This precipitate was removed by centrifugation at 12,000 x G for 15 minutes. Approximately 95% of the A_{260} material was recovered in the supernatant fraction (Figure 17).

(c) Discontinuous Sucrose Density Gradient Ultracentrifugation: The dextran sulfate-free supernatant was diluted appropriately with Buffer L to permit an efficient concentration of ribosomes by subsequent ultracentrifugation. If processing more than a kilogram of liver a second partition was introduced at this stage for further concentration of ribosomes. The diluted supernatant was centrifuged at 100,000 x G for 2 hours in a Type 42 fixed-angle rotor. The resulting supernatant was removed by aspiration and the gold-coloured ribosomal pellets were resuspended in Buffer L by gentle shaking. Homogenization of the pellet by mechanical shearing must be avoided at this step as disintegration of ribosome structure occurs and the yield of ribosomes is substantially reduced. The suspension was clarified by centrifugation at 23,000 x G for 30 minutes. The supernatant was centrifuged in a Type 42 fixed-angle rotor at 100,000 x G for 2 hours into discontinuous sucrose layers: 1.1 ml of 3 M and 5.0 ml of 2 M sucrose in Buffer L. By the end of centrifugation, the ribosomes were concentrated predominantly in the 2 M layer. Thus the danger of disrupting the ribosomes by mechanical suspension methods was eliminated. This ribosome fraction was the first material in the purification to have a maximum absorption at 257 nm (Figure 17).

(d) DEAE-Cellulose Column Chromatography of the Ribosome Fraction: The ribosome fraction obtained from the discontinuous sucrose gradient

FIGURE 17



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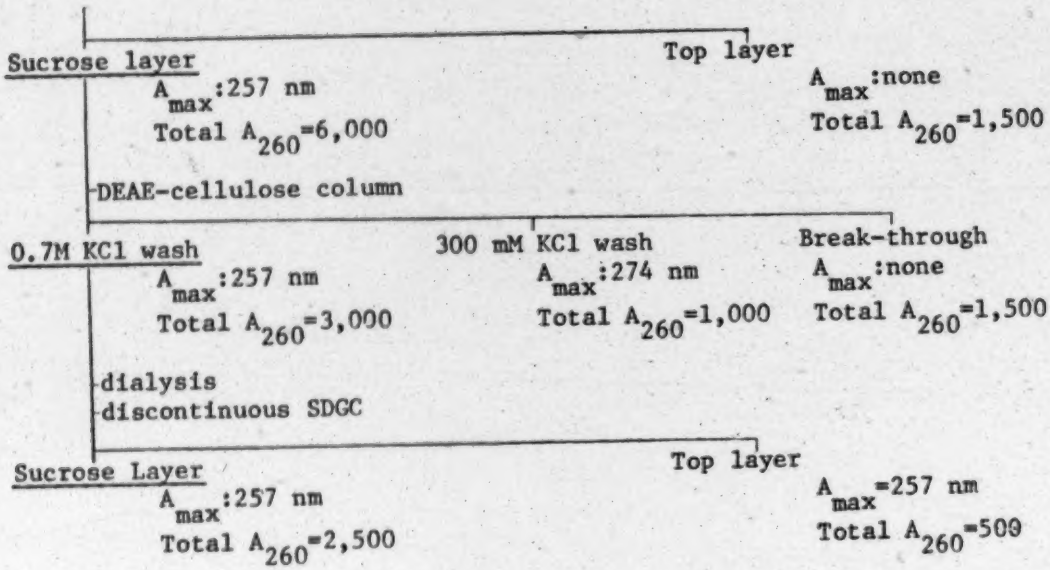


FIGURE 17: Schematic representation of the isolation procedure for rabbit liver ribosomes, including the change in optical profile and the yield of A_{260} units at each stage of the procedure. See section (i) for detailed description. *From 100 g of rabbit liver.

ultracentrifugation was diluted with Buffer L to reduce the salt concentration to less than 50 mM. This diluted fraction was then applied to a DEAE-cellulose column which had been equilibrated with Buffer L. The column was washed with Buffer L until the base line at 254 nm was attained on the UV monitoring system (ISCO). The column was then washed with Buffer L containing 300 mM KCl. The most significant feature of the 300 mM KCl wash was that it removed RNase, aminoacyl-tRNA synthetase and aminoacyl-tRNA:protein transferase from the ribosomes. The ribosomes were then eluted with Buffer L containing 0.7 M KCl. The recovery of A_{260} units in the break-through, 300 mM KCl wash and 0.7 M KCl wash fractions was approximately 25%, 15% and 50% respectively. The ratio of ribosomal and non-ribosomal materials at this stage of purification was constant for all preparations. The 0.7 M KCl elute was immediately dialyzed against 10 volumes of Buffer L for a minimum of 2 hours in order to reduce the K^+ concentration. The ribosomes were then concentrated by ultracentrifugation over 2 M and 3 M sucrose layers as described in Section (c). The final ribosomal suspension in the 2 M sucrose layer was adjusted to 100 A_{260} units per ml and stored in a liquid nitrogen refrigerator. The average yield of ribosomes was 2,500 A_{260} units per 100 g of rabbit liver.

(ii) Properties of Purified Rabbit Liver Ribosomes

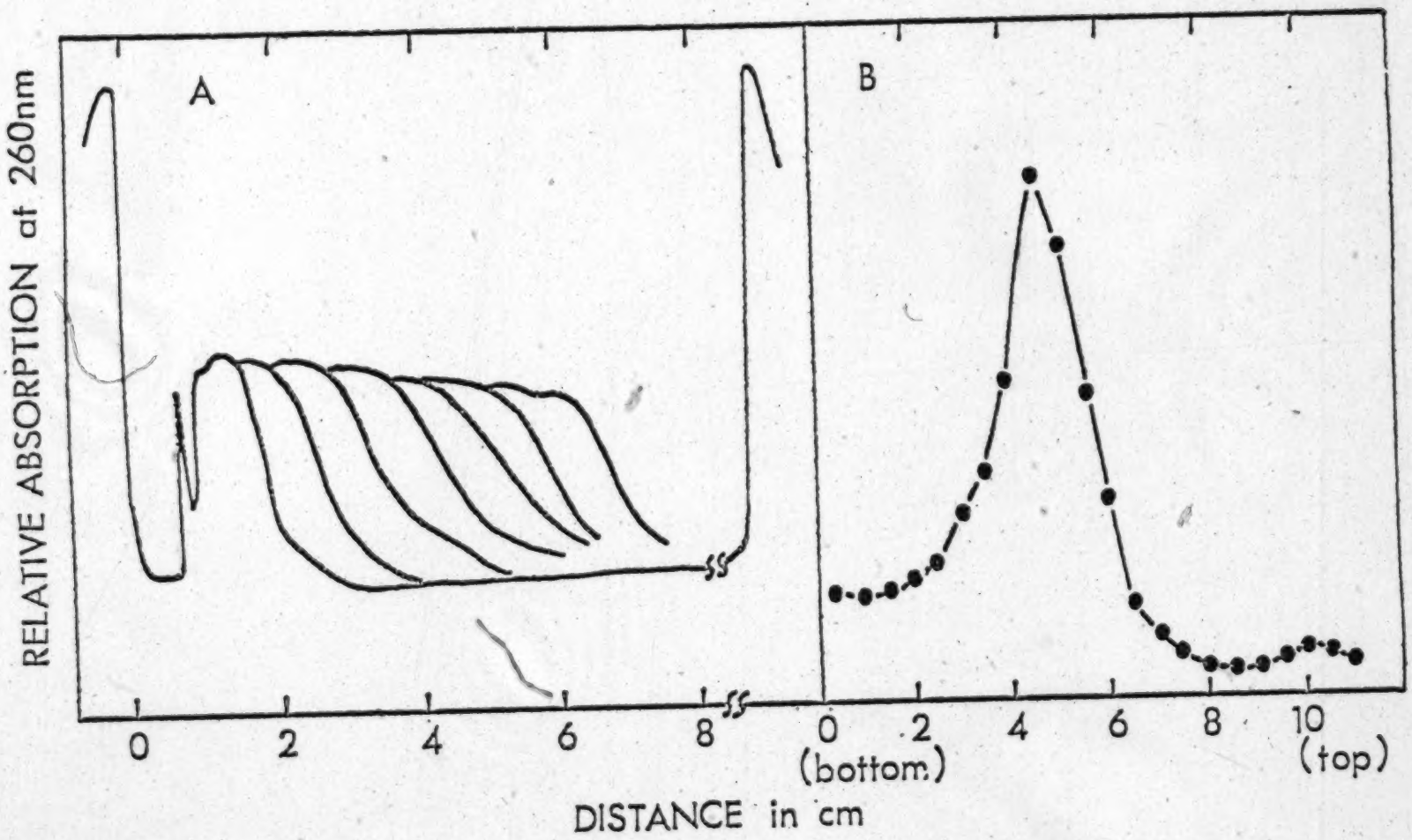
(a) Sedimentation Properties of the Purified Ribosomes: The sedimentation velocity boundary profile of the purified ribosomes was measured in a Beckman model E analytical ultracentrifuge as described in Chapter 2, Section III (xvii). The ribosome solution was diluted to 1.2 A_{260}.

The sample was spun at 40,000 rpm at 20°C. Photographs were taken at 2 minute intervals after the speed was attained. As seen in Figure 18A, the majority of particles sedimented as a single moving boundary. The sedimentation coefficient was calculated to be 78.0 ± 0.5S. This is close to the 80S value which is accepted as the typical value for mammalian ribosomes (215, 216).

The sedimentation profile of the purified ribosomes obtained by sucrose density gradient centrifugation (15% to 30%) at 180,000 x G for 2.5 hours was also examined. The results (Figure 18B) again indicated that the liver ribosomes moved as a single boundary, and that the preparation consisted of a homogeneous population of monoribosomes. No evidence was obtained for the presence of free ribosomal subunits.

(b) RNA and Protein Content of the Purified Ribosomes: To examine the variability in RNA and protein content of different preparations of rabbit liver ribosomes, the amount of RNA and protein in the purified ribosomes was estimated as described in Chapter 2, Section III (v). A stock of tRNA, purified according to a previously described method (217) was adjusted to 100 µg/ml and used as a standard in the RNA estimation. Similarly, bovine serum albumin was adjusted to 500 µg/ml and used as a standard in the protein estimation. An aliquot of the purified ribosome suspension was diluted to 5.0 A_{260} for both RNA and protein determinations. The results

FIGURE 18: Sedimentation profile of the purified rabbit liver ribosomes. (A) Spinco Model E analytical ultracentrifugation profile. The ribosome concentration was adjusted to give approximately 1.2 A_{260} units per ml of solution containing Tris-HCl (pH 7.0) 10 mM, KCl 100 mM and $MgCl_2$ 5 mM. The sample was run at 40,000 r.p.m. at 20°C. The photographs were taken at a wavelength of 265 nm at two min intervals after the speed was attained. The details of the method are presented in Chapter 2, Section III (xvii): (B) Sucrose density gradient centrifugation profile. Approximately 20 A_{260} units of purified ribosomes were loaded on a linear sucrose gradient (15 to 30%) in Buffer L, and were run at 180,000 g for 2.5 hr in the SW41 rotor of a Beckman L3-50 preparative ultracentrifuge.


RELATIVE ABSORPTION at 260nm
A
B
0
2
4
6
8
0
2
4
6
8
10
(bottom)
(top)
DISTANCE in cm

indicated that 1.0 A_{260} unit of the purified ribosomes contained 43.5 ± 1.5 µg of RNA by dry weight and 45.5 ± 1.5 µg of protein. Therefore, on the basis of these values, 1.0 A_{260} unit of ribosomes represents 90 µg by dry weight. This value is lower than those reported for ribosomes obtained by other methods. However, conventional methods used in other studies may not efficiently remove non-ribosomal proteins (218,220).

(c) Absence of RNase and Aminoacyl-tRNA synthetase in the Purified Ribosomes: For our proposed mRNA:ribosome complex formation studies it was essential that the ribosome preparation be free of RNase and aminoacyl-tRNA activities. RNase causes degradation of messenger RNA. The extent of hydrolysis varies so that the number of polynucleate fragments as well as the frame of the genetic codons becomes uncontrollable (162). The presence of aminoacyl-tRNA synthetase might create ambiguity in assessing aminoacyl-tRNA binding to the ribosome:mRNA complex, since Millipore filters usually retain aminoacyl-tRNA:synthetase complexes (221).

The kinetics of [^{3}H]-polyribonucleate hydrolysis by the purified rabbit liver ribosomes was examined as described in Chapter 2, Section III (xvi). In order to measure the sensitivity of the assay system the hydrolysis was also performed in the presence of a known concentration of pancreatic RNase A. The results (Figure 19) suggested that the assay system could detect the presence of 0.01 µg/ml of RNase. The purified ribosomes did not contain a detectable amount of RNase.

The aminoacyl-tRNA synthetase activity of the ribosome preparation

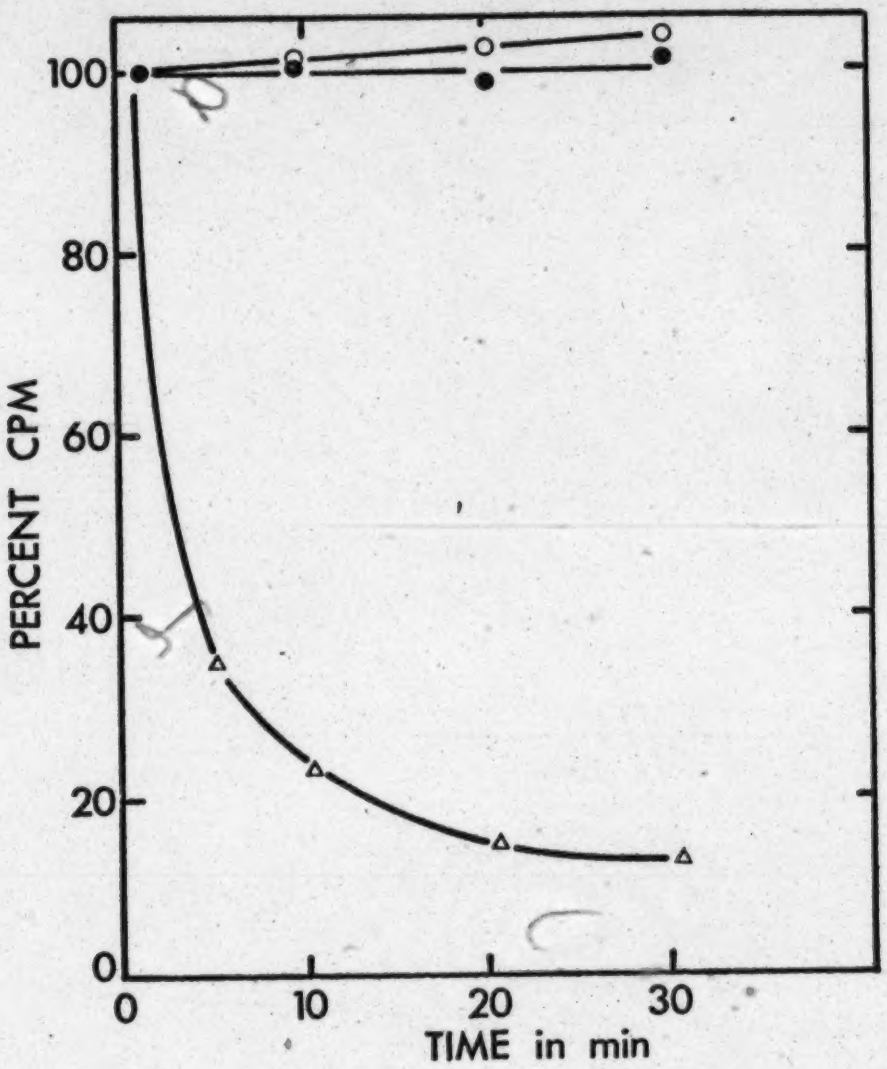


FIGURE 19: RNase activity of the purified rabbit liver ribosomes. Hydrolysis of [^{3}H]-polyribonucleates was measured in the presence of ribosomes (50 µg/0.1 ml reaction mixture) or pancreatic RNase A (concentrations as indicated) as described in Chapter 2, Section III (xvi).

Hydrolysis of [^{3}H]-polyuridylate by 50 µg of purified ribosomes (●);

Hydrolysis of [^{3}H]-polyadenylate by the purified ribosomes (O);

Hydrolysis of [^{3}H]-polyuridylate by pancreatic RNase A (0.01 µg/ml) (Δ).

was also examined as described in Chapter 2, Section III (xiii). Figure 20 illustrates the kinetics of phenylalanyl-tRNA synthesis by reticulocyte enzyme as compared to the ribosomal fraction. The concentration of reticulocyte protein added to the reaction was the same as that employed in the phenylalanine incorporation studies to be described in the following section. The results suggested that the ribosomal fraction contained only a negligible amount of phenylalanyl-tRNA synthetase activity. Additional evidence for the absence of aminoacyl-tRNA synthetases in the ribosome preparation was obtained from similar experiments using mixed amino acids.

Thus the absence of both RNase and aminoacyl-tRNA synthetase activities in the ribosome preparation was assured.

(d) Cell-Free Incorporation of Amino Acid into Protein: The purified rabbit liver ribosomes were ultimately to be used in the study of the mRNA:ribosome initiation complex formation. Before such studies could be undertaken it was necessary to ensure that the isolated ribosomes were biologically active. In order to assess this, amino acid incorporation in a cell-free system containing the liver ribosomes was carried out as described in Chapter 2, Section III (xiv). In this study, a specific comparison was made between ribosomal fractions prior to and after DEAE-cellulose chromatography, designated as pre- and post- DC ribosomes respectively. This comparison was made to determine the effect of DEAE-cellulose on the ability of the ribosomes to support cell-free protein synthesis. The results of such a study are presented in Figure 21. The results indicated that both ribosomal

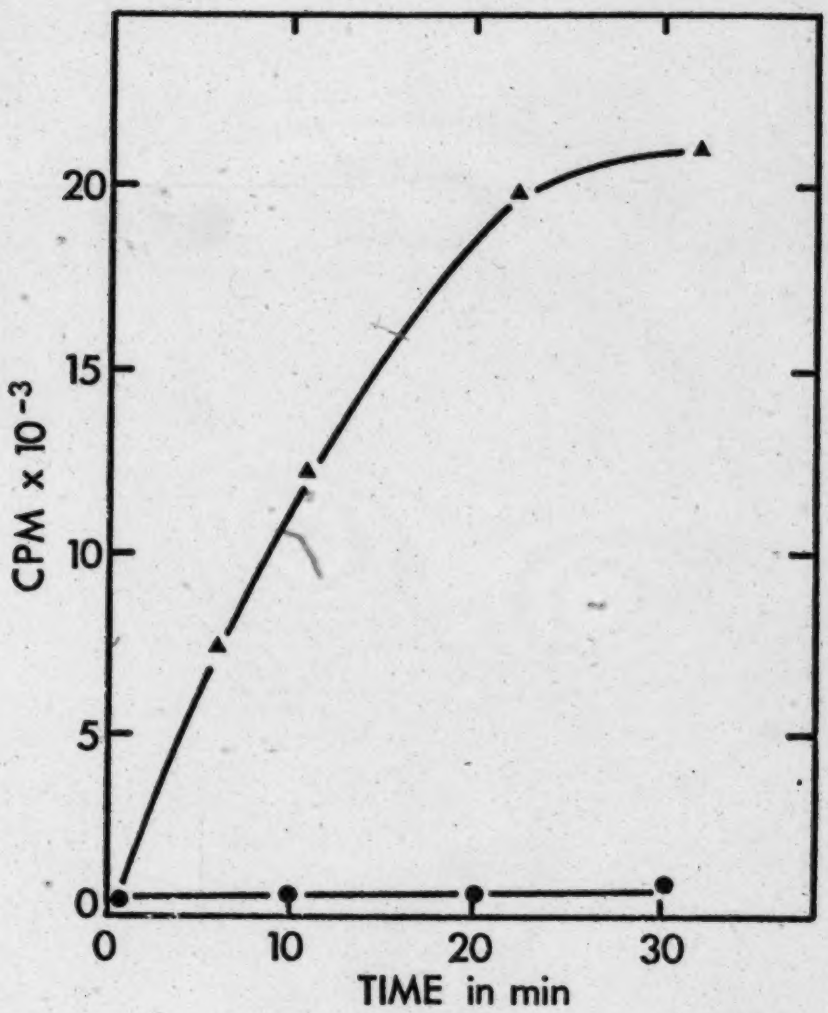


FIGURE 20: Phenylalanyl-tRNA synthetase activity of the purified ribosomes. The reaction conditions (in 0.5 ml) were described in Chapter 2, Section III (xiii). At intervals, 0.1 ml aliquots of reaction mixture were withdrawn and processed for radioactivity measurement (Chapter 2, Section III (i and iv)): [^{3}H]-phenylalanyl-tRNA synthesis by 50 µg of ribosomes in the absence of added reticulocyte enzyme (●); [^{3}H]-phenylalanyl-tRNA synthesis by 10 µg of reticulocyte enzyme in the absence of added ribosomes (▲).

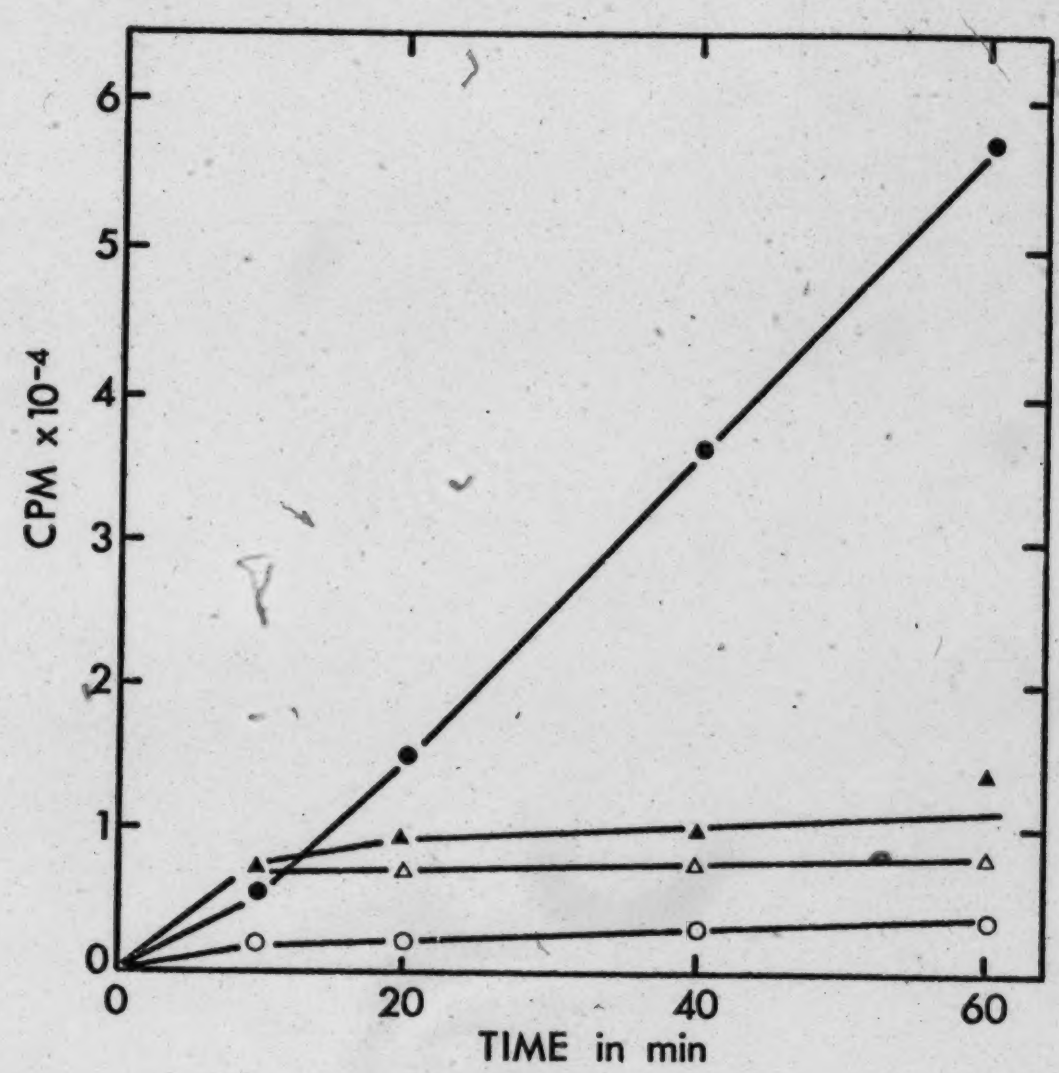


FIGURE 21: Cell-free [^{3}H]-polyphenylalanine synthesis by rabbit liver ribosomes of different purities. The reaction conditions were described in Chapter 2, Section III (xiv). At intervals, 0.1 ml aliquots of reaction mixture were withdrawn and processed by the hot TCA method for measuring radioactivity retained on each filter disc (Chapter 2, Section (i and iv)). [^{3}H]-phenylalanine incorporation by pre-DC ribosomes in the presence of polyuridylate (▲); by pre-DC ribosomes in the absence of added polyuridylate (△); by the post-DC ribosomes in the presence of polyuridylate (●); by the post-DC ribosomes in the absence of added polyuridylate(0).

fraction systems were able to support incorporation of [^{3}H]-phenylalanine into hot acid insoluble material in the presence of added polyuridylate. However, phenylalanine incorporation by the post-DC ribosome system increased linearly with time for the duration of the assay (60 minutes), whereas the incorporation by the pre- DC ribosome system decreased after 10 minutes of reaction. Since the only difference between the two assay system was in the quality of ribosomes added, the results suggested that DEAE-cellulose efficiently removed non-ribosomal materials which otherwise interfered with protein synthesis.

A comparison of the endogenous activity (protein synthesis in the absence of added mRNA) of the pre- and post- DC ribosomes was made. The results demonstrated that the pre-DC ribosomes exhibited 50% of the incorporation obtained in the presence of mRNA (50% endogenous activity). On the other hand, less than 10% endogenous incorporation was observed with the post-DC ribosomes. One interpretation of this result is that the post-DC ribosomes are free of aminoacyl-tRNA:protein transferase activity (212,222).

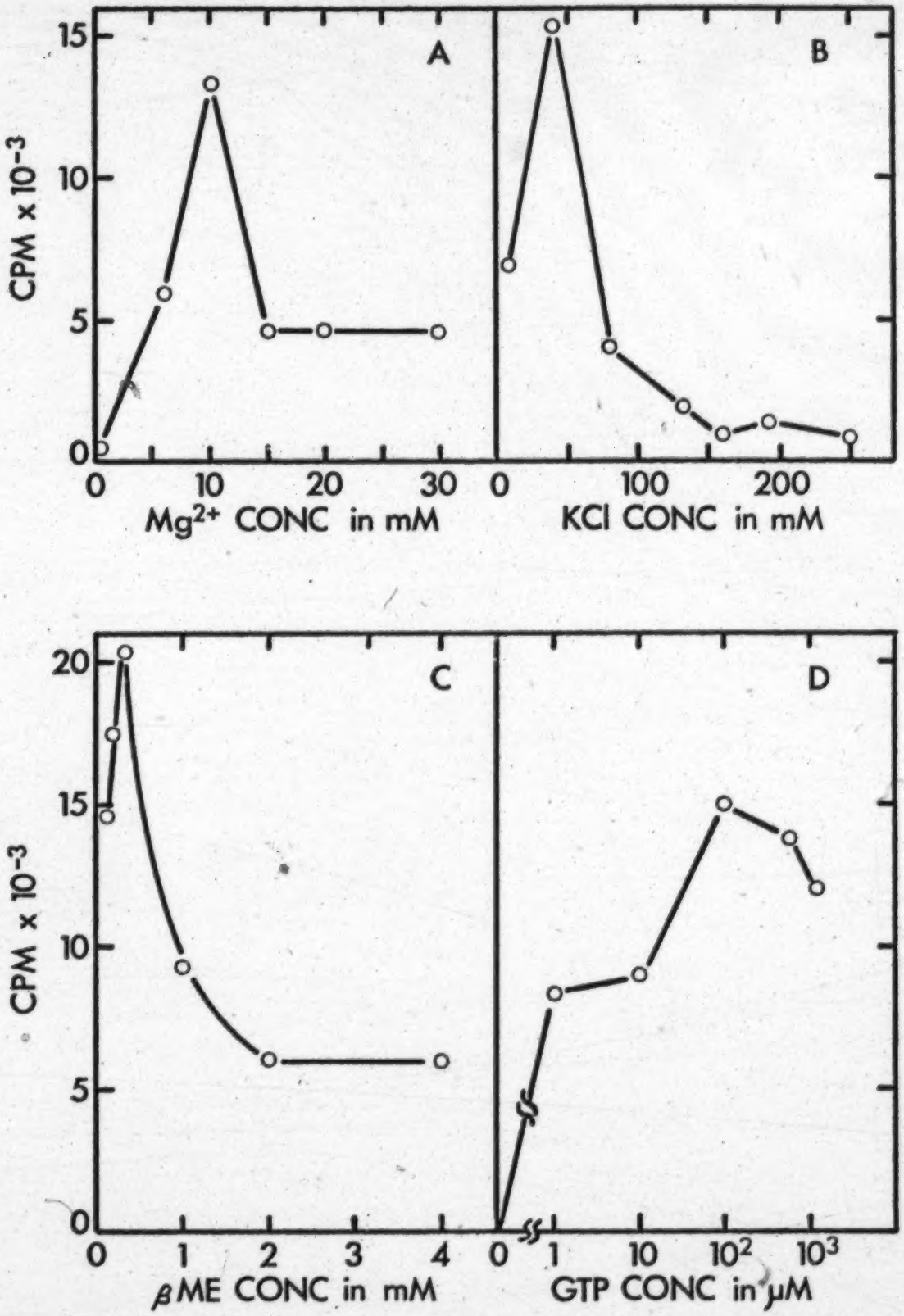
(e) Establishment of Optimum Reaction Conditions for Cell-Free Protein Synthesis by the Purified Liver Ribosomes: During the establishment of the rabbit liver ribosome system, the assay of biological activities had been monitored under the conditions normally applied to the bacterial system (155). This method, however, did not necessarily represent optimum conditions for the liver system. For this reason we decided to determine the optimum conditions for cell-free synthesis in

the rabbit liver ribosome system. This was accomplished by measuring the synthesis of [^{3}H]-phenylalanine in a cell-free protein-synthesizing system. In each set of experiments the concentration of only one of the reaction components was varied (Figure 22). When the concentration of Mg^{2+} was varied, it was found that the dependency of the reaction on Mg^{2+} was absolute, and that the optimum concentration was 11 mM (Figure 22A). At higher concentrations than 11 mM an inhibition was observed. K^+ concentration was also found to be critical (Figure 22B). The optimum KCl concentration was 40 mM. At higher or lower concentrations, inhibition of the reaction occurred. With respect to $MgCl_2$ and KCl, both the bacterial and the liver system possess similar ionic requirements for polyuridylate-directed phenylalanine incorporation (155,162).

The effect of β-mercaptoethanol concentration was found to be very different than that in the bacterial system (Figure 22C). In the bacterial system, concentrations between 4 and 20 mM β-mercaptoethanol are usually employed for cell-free protein synthesis. In the rabbit liver ribosome system, however, 60% inhibition was observed in polyuridylate directed phenylalanine incorporation even at 2 mM β-mercaptoethanol. The optimum β-mercaptoethanol concentration was in the range of 0.2 to 0.4 mM. The mechanism of this inhibition by β-mercaptoethanol is not understood.

The dependency of the reaction on GTP was absolute in the rabbit liver ribosome system (Figure 22D), as expected in any cell-free protein synthesizing system. Although the optimum concentration of GTP for polyuridylate-directed phenylalanine incorporation was 0.1 mM, increasing

FIGURE 22: Optimum reaction conditions for cell-free [^{3}H]-polyphenylalanine synthesis by the purified rabbit liver ribosomes. The reaction was carried out as described in Chapter 2, Section III (xiv). (A) $MgCl_2$ concentration was varied; (B) KCl concentration was varied; (C) β-mercaptoethanol concentration was varied; (D) GTP concentration was varied. For each experiment the samples were processed for radioactivity measurement as described in Chapter 2, Section III (i and iv).


A
B
C
D
CPM x 10⁻³
15
10
5
0
20
0
10
20
30
100
200
1
2
3
4
10
10²
10³
Mg²⁺ CONC in mM
KCl CONC in mM
βME CONC in mM
GTP CONC in μM

the GTP concentration had little effect on the extent of the incorporation. The liver ribosomal system also required a relatively low concentration of ATP (0.2 mM).

The effect of pH on the reaction was also examined and the optimum pH was 7.8. Lower pH conditions decreased the incorporation drastically, whereas pH greater than 9.0 did not result in such a drastic effect.

(f) Absence of mRNA Binding Capacity of the Purified Rabbit Liver Ribosomes: Before studies into mRNA:ribosome complex formation could be initiated, it was necessary to ensure that the ribosomes were biologically active. This was accomplished by demonstrating the ability of the ribosomes to support cell-free incorporation of amino acid into protein as described in Section (d). The preparation of biologically active ribosomes from rabbit liver provided us with a full complement of components originally thought necessary to study both mRNA:ribosome and aminoacyl-tRNA:mRNA:ribosome complex formations. Having obtained these components we focused our attention on the mechanism of mRNA:ribosome complex formation.

The binding ability of the purified ribosomes for synthetic mRNA's was examined as described in Chapter 2, Section III (xv). To our surprise, the purified ribosomes were totally inactive in the binding of poly-A,-C, -G, and -U as determined by Millipore filtration (Chapter 3, Section III (xv)). However, according to our previous studies, these same ribosomes were capable of supporting polyuridylate-directed polyphenylalanine synthesis when supplemented with crude protein from a post-

ribosomal fraction. This latter ability indicated that mRNA:ribosome complex formation must have taken place under the cell-free protein-synthesizing conditions and that possibly this formation was being mediated by some component(s) present in the crude protein fraction. Thus the inability of the purified ribosomes to bind the synthetic mRNA's suggested that the mRNA binding capacity of the active ribosomes was mediated by some factor(s), ribosomal or non-ribosomal, which became separated from the ribosomal fraction during the purification procedure. This possibility was strengthened by the fact that a complete removal of mRNA binding capacity from ribosomal fractions had been reported in the E. coli ribosome system (162). In order to determine at which stage of the purification scheme the rabbit liver ribosome fraction lost the ability to bind mRNA, [^{3}H]-poly-U binding to various fractions during the course of ribosome preparation were examined using the Millipore filtration method (Chapter 2, Section III (xv)). The results of these studies are presented in Table 9. The results indicated that complete removal of poly-U binding capacity from the ribosomes occurred at the very last stage of purification; that is, at the step of DEAE-cellulose column chromatography and subsequent concentration of ribosomes by ultracentrifugation. Although complete removal of the binding activity occurred at the final stage of purification, the majority of binding capacity was released in the first ultracentrifuge supernatant fraction (UC-I fraction). These facts supported the possibility that mRNA binding to the rabbit liver ribosomes was mediated by an extra-ribosomal protein factor(s).

TABLE 9

Change in poly-U Binding Capacity of Ribosomes

During the Purification Procedure

Purification Step	[^{3}H]-poly-U Bound/A_{260} Units	
	Ribosomal Fraction	Non-ribosomal Fraction
1st ultracentrifugation of Dextran sulfate layer	32,400 ($2.43 \times 10^{8*}$)	16,600 ($1.17 \times 10^{8*}$)
2nd ultracentrifugation	36,400 (2.18×10^{8})	1,970 (3.00×10^{6})
DEAE-cellulose chromatography	16,300 (4.90×10^{7})	45,800 (4.58×10^{7})
Condensation of ribosomes by ultracentrifugation	2,810 (7×10^{6})	17,900 (1×10^{7})
1 M KCl wash of ribosomes	100 (-)	(-) (-)

* Total activity

III. Discussion

The preparation method described in this chapter gives rise to a large quantity of biologically-active rabbit liver ribosomes. The ribosomes obtained from each preparation are of similar quality, a feature which makes them particularly suitable for structural and functional studies. The reproducibility in obtaining ribosomes of similar quality can be judged in terms of sedimentation properties, protein-RNA ratio, lack of lytic enzyme activity, and lack of mRNA binding as well as aminoacyl-tRNA binding capacities. A critical feature of the procedure is that the ribosomes are exposed to high salt (KCl) concentration twice during the preparation, once during the partition step and again during the DEAE-cellulose chromatography step. The short exposure to high salt at the partition step does not cause any noticeable effect on the yield of the quality of ribosomes. However, the second exposure to high salt at the DEAE-cellulose chromatography step is critical in two aspects: (1) the 0.7 M KCl wash-fraction contains ribosomes. If this fraction is concentrated directly by ultracentrifugation dissociation of the ribosomes occurs. The resulting ribosomal subunits remain polydispersed in the top layer of the discontinuous sucrose gradient. Dissociation of high-salt treated ribosomes into subunits in the centrifugal force field has been reported previously (223-225). This phenomenon causes reduction in the final yield of ribosomes. In order to avoid this situation, it is necessary to dialyze the 0.7 M KCl wash fraction against a low-salt buffer to lower the salt concentration below 400 mM prior to ultracentrifugation (2). The exposure of the

ribosome fraction to 0.7 M KCl during the DEAE-cellulose chromatography step removes the mRNA binding capacity from the ribosomes. This represents a unique feature of the purification procedure, one which subsequently led us to the discovery of mRNA binding factors.

With the preparation of biologically active ribosomes from rabbit liver, the fourth requirement toward establishment of an homologous system was accomplished. However, the inability of the purified ribosomes to bind mRNA, necessitated that the factor(s) responsible for mediating mRNA binding to the ribosomes had to be isolated and characterized.

CHAPTER 6

PURIFICATION AND CHARACTERIZATION OF mRNA BINDING FACTORS

I. Introduction

In Chapter 5 it was demonstrated that purified rabbit liver ribosomes could support polyuridylate-directed [^{3}H]-polyphenylalanine synthesis when supplemented with a crude post-ribosomal protein fraction, but that the ribosomes themselves were unable to bind synthetic mRNA's such as poly-A, -C, -G, and -U. These facts suggested that the mRNA binding capacity of the active ribosomes was mediated by some factor(s), ribosomal or non-ribosomal, which became separated from the ribosomes during the purification procedure. Examination of the [^{3}H]-poly-U binding capacity of various fractions throughout ribosome purification demonstrated that the majority of binding capacity was released in the first ultracentrifuge supernatant fraction. This fraction, UC-I fraction, was subsequently found to be a plentiful source for all four synthetic mRNA binding factors. The mRNA binding proteins have been designated as M_A-, M_C-, M_G-, and M_U- factors and are specific to poly-A, poly-C, poly-G and poly-U respectively. Although four mRNA binding factors were isolated, only M_A- and M_U- factors have been purified to homogeneity. Unfortunately M_C- and M_G- factors are so unstable that they have not been purified to date. The purification and characterization of the mRNA binding factors, M_A and M_U, is the subject of this chapter. In particular, the role of these factors in the binding of synthetic mRNA's to ribosomes is examined.

The purification of the mRNA binding factors includes conventional

chromatography on DEAE-cellulose and Sepharose 6B. Although M_A- and M_U- factors exhibit the same molecular weight, 60,000 daltons, as determined by SDS-gel electrophoresis, they elute from DEAE-cellulose column at different salt concentrations and require different ionic environments to maintain their mRNA binding capacity. Both factors mediate the specific binding of the corresponding synthetic mRNA to the purified rabbit liver ribosomes in a completely homologous cell-free system. The ternary complex of mRNA:ribosome:M-factor was then able to bind with a specific aminoacyl-tRNA when an additional aminoacyl-tRNA binding factor was added to the reaction mixture. Therefore it seems that the observed function of M_A- and M_U- factors represents a biologically significant event and not an artifact.

Of the known factors involved in the initiation of mammalian protein synthesis (see Chapter 1, Table 1) M_A- and M_U- factors most closely resemble initiation factor IF-M_3 in terms of function (135). However, their molecular weight differs considerably from the known value for IF-M_3. Moreover, one of the unique properties of the M-factors, which is not comparable to other known factors, is that each factor is specific to a homopolynucleate. This fact suggests that some mechanism of base recognition by protein may be involved in the present system. In this regard, it is unfortunate that the lack of purified M_C- and M_G- factors prevents a study on recognition of natural mRNA by the M-factors.

II. Results

(1) Purification of mRNA Binding Factors

Prior to description of the purification methods, some important

aspects of the approach taken should be brought to attention. The protein factors for mRNA binding to ribosomes can be isolated from either one of two major sources: the post-ribosomal supernatant or the 1 M KCl wash of crude ribosomes. As illustrated in Table 10, the mRNA binding factors in the ribosome wash fraction are extremely unstable after exposure to a high-salt buffer. The reason for this differential stability of binding factors from these two sources will be discussed in a subsequent section (Figure 24). It should be pointed out that four mRNA binding factors were detected in the crude protein fractions, however the poly-C and poly-G binding factors were too unstable to be purified under the conditions employed. It should also be mentioned that poly-A and poly-U binding factors can be stabilized and purified from the post-ribosomal supernatant under different conditions, as will become evident in the following sections. During the purification procedure, the mRNA binding capacities of each fraction were monitored by the Millipore filtration method as described in Chapter 2, Section III (xv).

(a) DEAE-cellulose Chromatography of Post-Ribosomal Proteins: Post-ribosomal proteins, UC-I fraction, were obtained as described in Chapter 5 for the preparation of rabbit liver ribosomes. In order to precipitate proteins, solid ammonium sulfate was added with stirring. The proteins precipitating at a saturating concentration of ammonium sulfate were collected by centrifugation at 12,000 x G for 30 minutes. The supernatant was collected by aspiration and discarded. The precipitate was suspended in a minimum volume of Buffer TR1 (see Chapter 2, Section II (ii)), and stored at -20°C. When required, a 2.5 g portion of protein

in the ammonium sulfate precipitate of the UC-I fraction (Table 10) was dialyzed against 40 volumes of Buffer TR1. The salt concentration of the dialysate was adjusted to 50 mM K^+ equivalent, as measured by a conductivity meter. This fraction was then applied to a DEAE-cellulose column (3.7 cm x 37 cm) which had been equilibrated with Buffer TR1. The column was first washed with Buffer TR1 containing 50 mM KCl until the base line at 280 nm on a UV monitoring system (ISCO) had been attained. Subsequently, the column was developed with a 1600 ml, linear KCl gradient from 0 to 500 mM in Buffer TR1. The poly-A and poly-U binding activities of each fraction were examined. Fractions showing significant binding activities were pooled (Figure 23). Poly-A binding activity appeared at approximately 170 mM KCl and poly-U binding activity at 220 mM KCl.

(b) Differential Dialysis and Re-chromatography of Each Factor: The two fractions corresponding to poly-A and poly-U binding activities respectively were subjected to differential dialysis. The poly-A binding fraction was dialyzed against Buffer TR1 containing 150 mM ammonium sulfate and 2.4 M sucrose for 10 hours. The poly-U binding fraction was dialyzed against the buffer containing only 2.4 M sucrose The reason for this differential treatment was that each binding factor was found to exhibit different stabilities in salt solutions as illustrated in Figure 24. The poly-A binding factor was found to be stable in buffer containing 150 mM ammonium sulfate, but unstable in solutions containing no salt. On the other hand, the poly-U binding factor was found to be unstable in buffer containing 150 mM ammonium sulfate, but stable in a salt-free solution. Therefore, the small amount of the poly-U binding

TABLE 10

Distribution of mRNA Binding Factors in Two Major Sources: the post-ribosomal supernatant, and the high-salt wash of crude ribosomes

Fraction	Messenger RNA Binding Activity*			
	Poly-A Binding	Poly-G Binding	Poly-C Binding	Poly-U Binding
Post-ribosomal supernatant (UC-1 Fraction)	1.6×10^4 (1.6×10^8)**	1.1×10^4 (1.1×10^8)	1.3×10^3 (1.3×10^7)	7.7×10^3 (7.7×10^7)
$(NH_4)_2SO_4$ precipitate of UC-I Fraction	2.0×10^4 (2.0×10^8)	3.4×10^3 (3.4×10^7)	not detectable	1.3×10^4 (1.3×10^8)
High-salt wash of crude ribosomes	1.8×10^4 (3.1×10^4)	8.0×10^4 (1.4×10^6)	7.3×10^3 (1.3×10^5)	6.3×10^5 (1.1×10^7)
$(NH_4)_2SO_4$ precipitate of ribosome wash	not detectable	not detectable	not detectable	7.0×10^5 (1.21×10^7)

* Expressed as [^{3}H]polynucleate bound (CPM) per mg protein.

** Numbers in brackets represent a calculated total activity in each fraction.

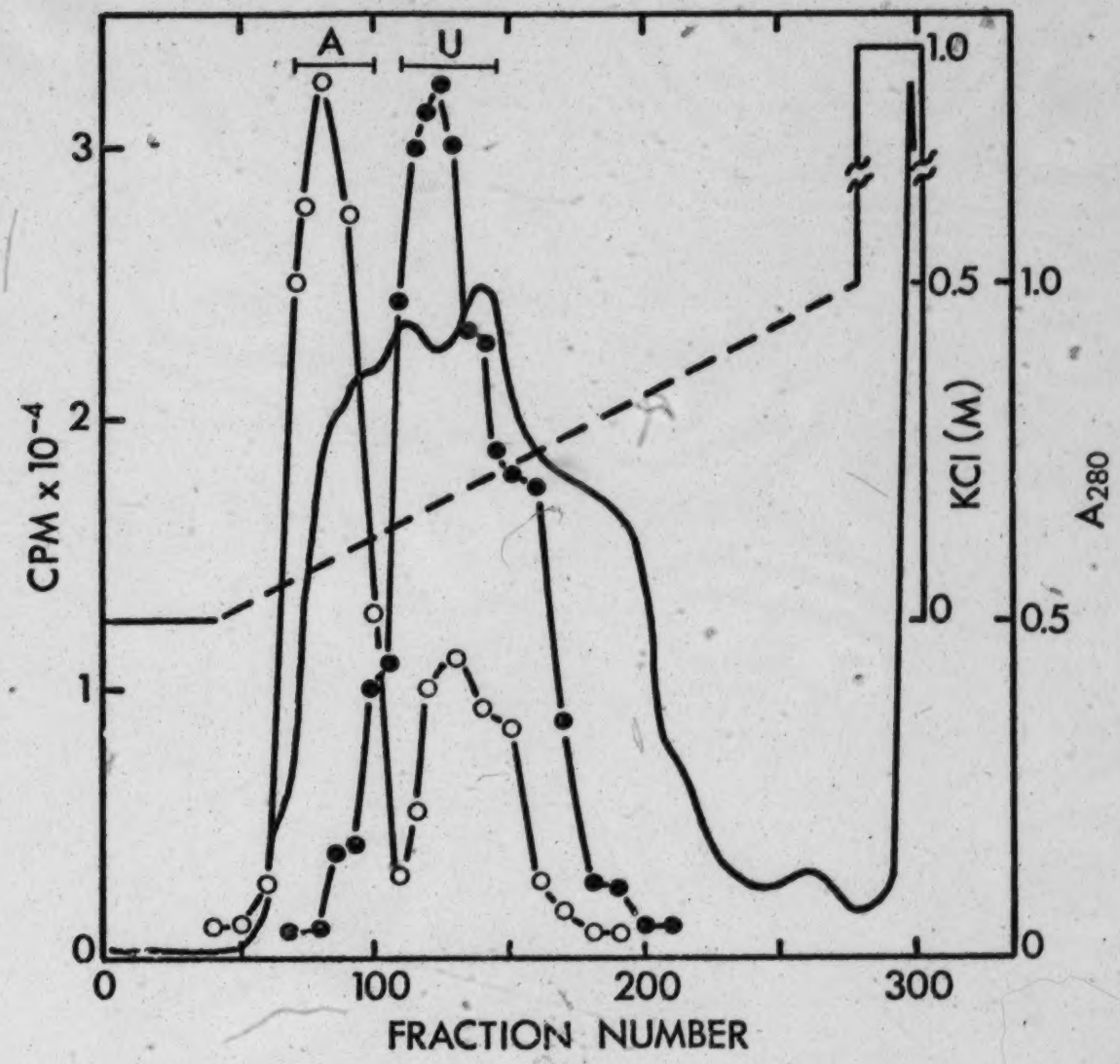


FIGURE 23: DEAE-cellulose column chromatography of the post-ribosomal supernatant proteins for the isolation of poly-A and poly-U binding factors. A 2.5 g portion of post-ribosomal supernatant proteins was loaded onto a DEAE-cellulose column (3.7 cm x 37 cm). The proteins were fractionated by a linear gradient of KCl from 0 to 500 mM in Buffer TR1. Then 6 ml was collected in each tube, and 100 µl aliquots were assayed for [^{3}H]-polynucleate binding activity by the Millipore filtration method as described in Chapter 2, Section III (xv). Active fractions for the binding of poly-A (tube no. 70-100) and poly-U (tube no. 110-145) were pooled, as indicated in the Figure by A. and U. A solid line indicates 280 nm absorption recording by the ISCO UV-Analyzer; poly-A binding (O), poly-U binding (●). The KCl gradient is inserted in the figure.

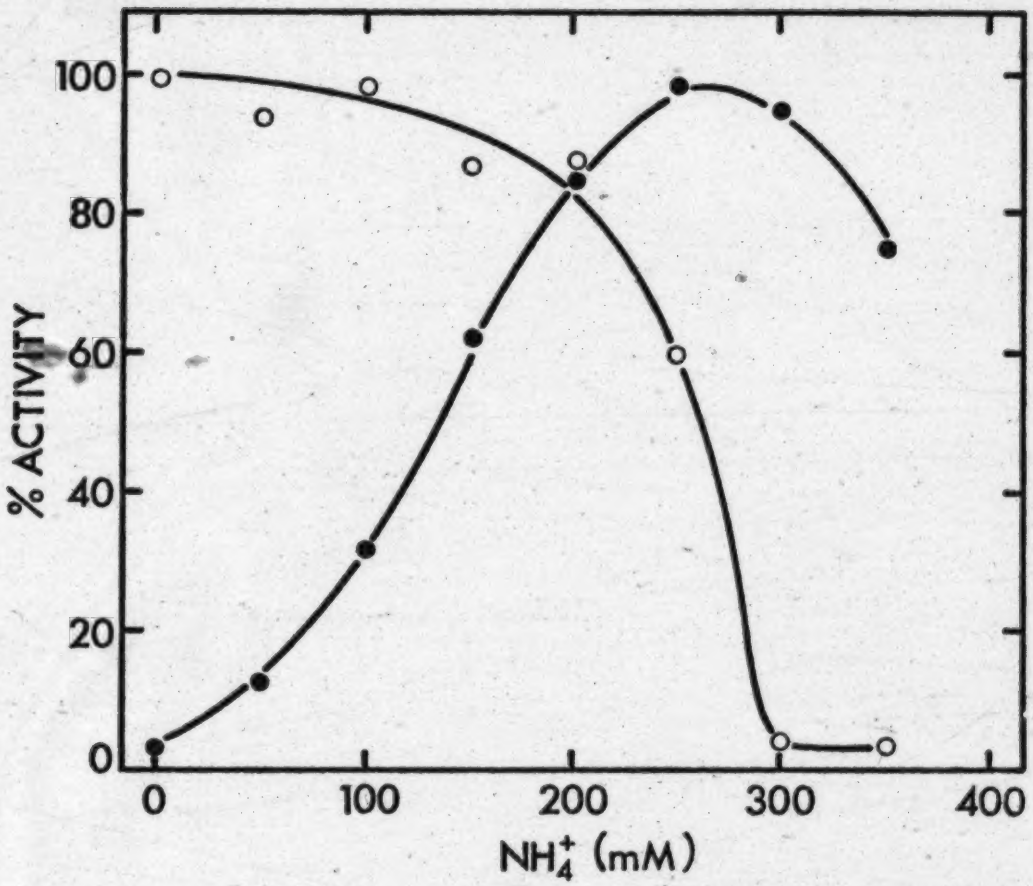


FIGURE 24: Inactivation profiles of poly-A and poly-U binding factors by salt in a buffer. 10 µg portions of purified poly-A and poly-U binding factors were individually incubated in Buffer TR1 containing different amounts of NH_4^+ (ammonium sulfate) as indicated on the abscissa. After 16 hr of standing at 4°C, the binding capacity was examined by the Millipore filtration method (Chapter 2, Section III (xv)). The results are expressed as percent activity of [^{3}H]-poly-A (●) and [^{3}H]-poly-U (0) binding by the non-treated poly-A binding factor and non-treated poly-U binding factor respectively. A 100% value is equivalent to 15,000 c.p.m.

factor present in the poly-A binding factor fraction was inactivated during the dialysis against the buffer as specified. This feature permitted separation of each factor, free from the other, at the very early stage of the purification procedure. It should be pointed out that conditions which eliminated one mRNA binding capacity did not result in a concomitant increase in the other mRNA binding capacity. At no time during the purification procedure was interconversion of mRNA binding capacities observed; that is, each binding capacity remained specific to the corresponding mRNA throughout the purification procedure.

After differential dialysis each fraction was rechromatographed on DEAE-cellulose columns until the protein peak coincided with the activity peak of the mRNA binding. This step usually required two successive chromatographies under conditions similar to those described for the first DEAE-cellulose chromatography. The elution profiles for these purification procedures are presented in Figure 25.

(c) Sepharose 6B Chromatography: After obtaining mRNA binding factors with a homogeneous charge property, each factor was subjected to a molecular sieving chromatography using Sepharose 6B (150 ml bed volume). In order to eliminate any interference by the inherent charge property of the Sepharose, the developing buffer contained 150 mM ammonium sulfate. Figure 26 illustrates the elution profiles of poly-A and poly-U binding factors from the column.

In order to obtain an approximate molecular weight for the binding factors, a mixture of four marker proteins (thyroglobulin, glucose

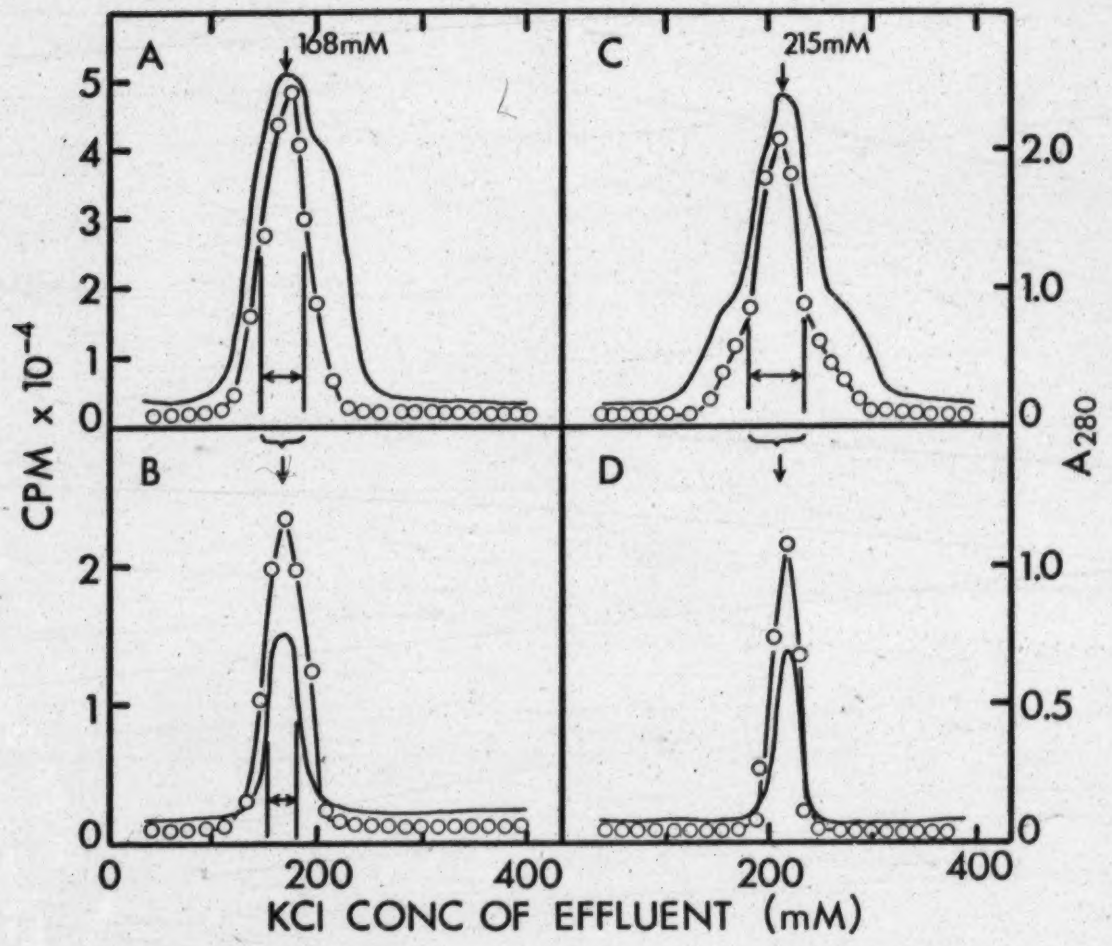


FIGURE 25: Purification of poly-A and poly-U binding factors by re-chromatography on DEAE-cellulose column. The pooled fractions, A and U, as indicated in Fig. 23 were separately chromatographed on DEAE-cellulose columns. (A) and (B) represent purification profiles of the poly-A binding factor: (A) second chromatography on a 50 ml column with 400 ml of a linear gradient of KCl from 0 to 400 mM; (B) third chromatography on a 10 ml column with 200 ml of a linear gradient of KCl from 0 to 400 mM. Two ml fractions were collected in test tubes, and 30 µl aliquots were assayed by the Millipore filtration method (Chapter 2, Section III (xv)) for the binding of [^{3}H]-poly-A. (C) and (D) represent purification profiles of the poly-U binding factor: (C) second chromatography with the same elution and assay conditions as in (A), except assay using [^{3}H]-poly-U; (D) third chromatography, as (B), except assay using [^{3}H]-poly-U. Solid lines represent the recording of 280 nm absorption by the ISCO UV analyzer.

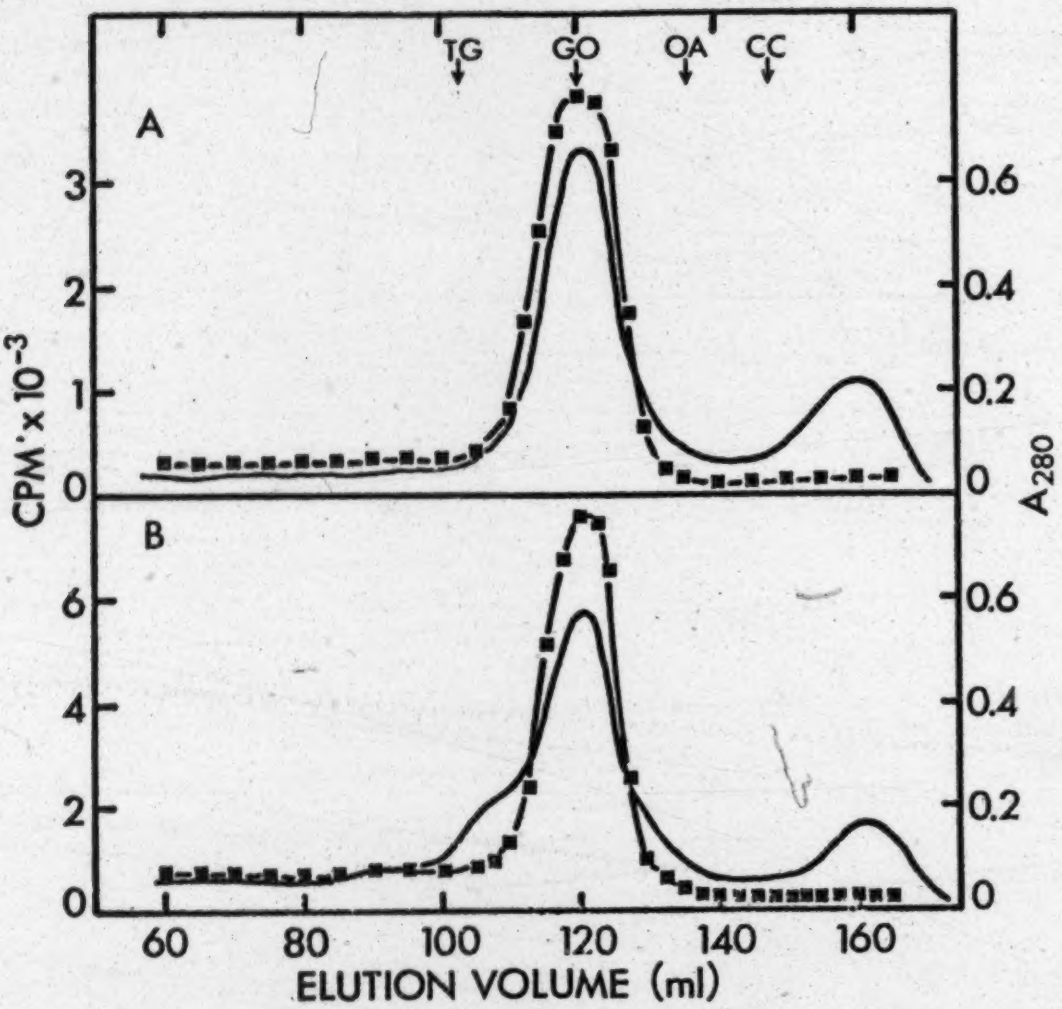


FIGURE 26: Sepharose 6B chromatography of poly-A and poly-U binding factors. Poly-A and poly-U binding factors were recovered from the active fractions, as indicated by arrows in Fig. 23, (C) and (D) respectively. Pooled fractions were concentrated by dialysis against appropriate buffers (see Fig. 24), and subjected to molecular sieving chromatography on Sepharose 6B, bed volume 150 ml. The columns were developed with Buffer TR1 containing 150 mM ammonium sulfate to counteract with charges on the Sepharose. One ml fractions were collected in each tube, and 20 µl aliquots were assayed for [^{3}H]-mRNA binding activity by the Millipore filtration method (Chapter 2, Section III (xv)). (A) represents poly-A binding factor from Fig. 25B and (B) poly-U binding factor from Fig. 25D.

After each chromatography, a mixture of four marker proteins were processed. The elution positions of these markers are illustrated by TG (thyroglobulin, MW = 6.6 x 10^5); GO (glucose oxidase, MW = 1.86 x 10^5); OA (Ovalbumin, MW = 4.5 x 10^4); and CC (cytochrome C, MW = 1.24 x 10^4). Estimated molecular weights of poly-A and poly-U binding factors are the same: approximately 180,000.

oxidase, ovalbumin, and cytochrome c) was applied to the column under identical conditions as those used for the binding factors. These marker proteins were run singly and in combination in order to ensure that no aggregation occurred between the proteins. The marker positions are illustrated in Figure 26. The poly-U binding activity appeared at the protein peak corresponding to a molecular weight of 180,000 (Figure 26A). At the region of low molecular weight quantity of protein was detected. This fraction included RNase as determined by the method described in Chapter 2, Section III (xvi). The poly-A binding activity appeared at the same position as the poly-U binding factor (Figure 26B). These facts indicated that both binding factors possess approximately the same molecular weights although they possess different net negative charges as demonstrated by DEAE-cellulose column chromatography.

Each binding factor obtained from Sepharose 6B chromatography was condensed by dialysis against Buffer TR1 containing 2.4 M sucrose and an appropriate concentration of salt: 150 mM ammonium sulfate for poly-A binding factor, and no salt for poly-U binding factor. The concentrated mRNA binding factors, designated as M_A- and M_U- factors were stored at -20°C.

(ii) SDS-Gel Electrophoresis

In order to determine the homogeneity of the purified M- factors, M_A- and M_U- factors were subjected to SDS-gel electrophoresis at pH 7.1 as described in Chapter 2, Section III (xviii). Figure 27 illustrates a densitometric tracing of such gels. The results indicated that

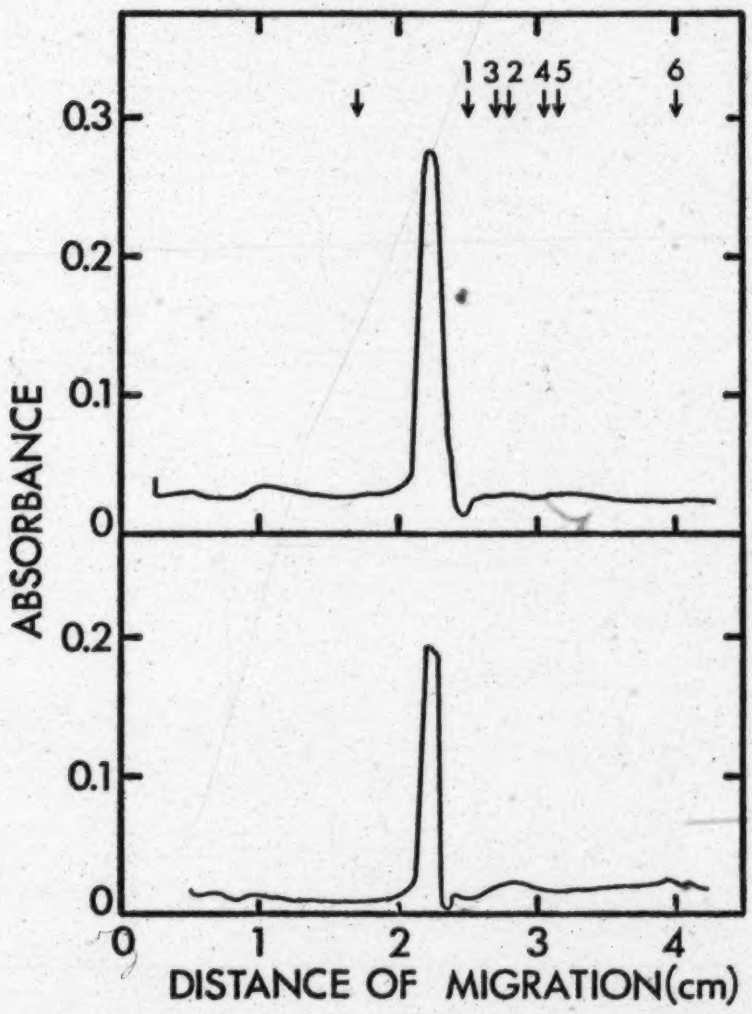


FIGURE 27: Densitometric tracing of SDS-gel after electrophoresis of M-factors. 10 µg of each purified M-factor, M_A and M_U, was treated with SDS (2%) at 85°C for 15 min in the presence of 50 mM dithiothreitol. Then the sample was subjected to electrophoresis as described in Chapter 2, Section III (xviii). The gel was stained with Coomassie brilliant blue and then destained. The densitometric tracings were made using an ISCO gel scanner Model 659:(A) M_A-factor; (B) M_U-factor. The relative positions of marker proteins which were treated exactly the same way are indicated by arrows with numbers: 1, Ovalbumin (MW = 4.5×10^4; 2. Aldolase subunits (MW = 4.0×10^4); 3. Succinyl-CoA synthetase β subunit (MW = 3.9×10^4); 4. Succinyl-CoA synthetase α subunit (MW = 2.85×10^4); 5. Chymotrypsinogen (MW = 2.75×10^4); 6. RNase A (MW = 1.37×10^4).

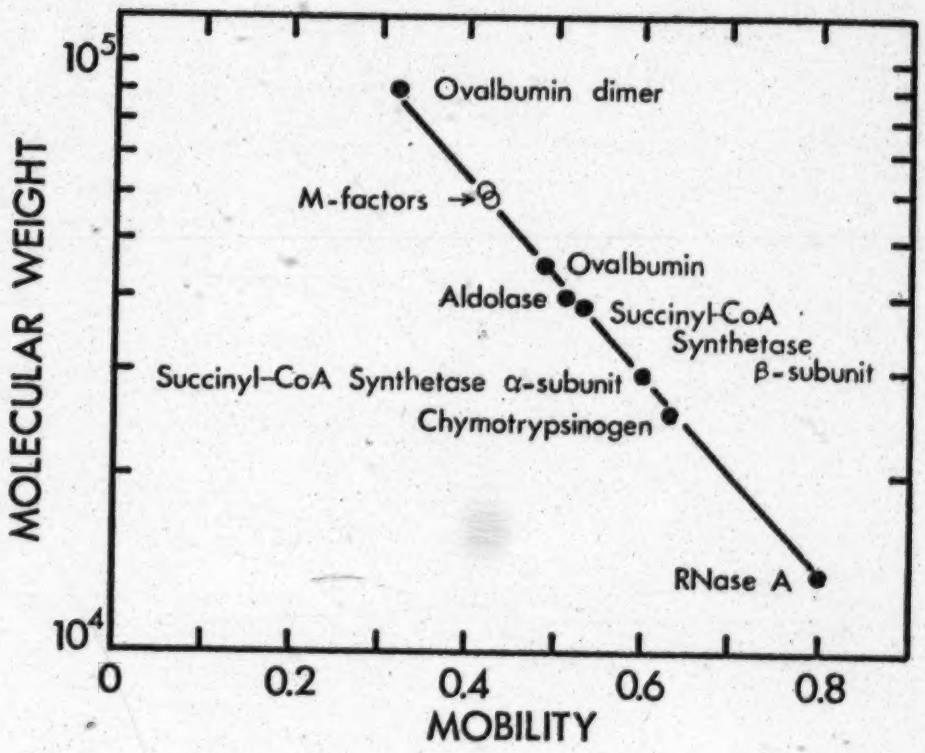


FIGURE 28: Molecular weight determination of poly-A and poly-U binding factors by acrylamide gel electrophoresis in SDS (Chapter 2, Section III (xviii)). The positions of the M-factors are indicated by M_A-factor and M_U-factor. Both factors indicate a molecular weight of approximately 60,000. The mobility of succinyl-CoA synthetase was used as an internal standard.

purified M_A- and M_U- factors each give rise to a single band in the presence of SDS. Calculation of the molecular weight of M_A- and M_U- factors by comparison to the relative mobilities of several standard proteins (Chapter 2, Section III (xviii)), suggested a value of 60,000 for both M-factors (Figure 28).

(iii) M-Factor Mediated Binding of mRNA to Ribosomes

In the following studies on mRNA:ribosome complex formation, ribosomes active in phenylalanine incorporation yet inactive in [^{3}H]-mRNA binding in the absence of added mRNA binding factor were used. It should be pointed out that when ribosomes possessed residual binding activity, they were subjected to dialysis against Buffer TR1 containing no salt and subsequently against the buffer containing 150 mM ammonium sulfate. This treatment inactivated the residual binding capacity of the ribosomes as described in the preceeding section. The biological activity of these ribosomes was examined by the incorporation of [^{3}H]-phenylalanine in the presence of a crude protein mixture from a post-ribosomal supernatant fraction.

In the study of mRNA:ribosome complex formation both Millipore filtration and sucrose density gradient centrifugation methods were used (Chapter 2, Section III (xv). The latter method was used to test whether or not the purified ribosomes could bind [^{3}H]-polyadenylate in the presence of the poly-A binding factor or to bind [^{3}H]-polyuridylate in the presence of the poly-U binding factor. The results of such sucrose density gradient centrifugation experiments (Figure 29) clearly demonstrated that ribosomes alone cannot bind poly-A or poly-U. Moreover, the ribosomes were not able to bind poly-A in the presence

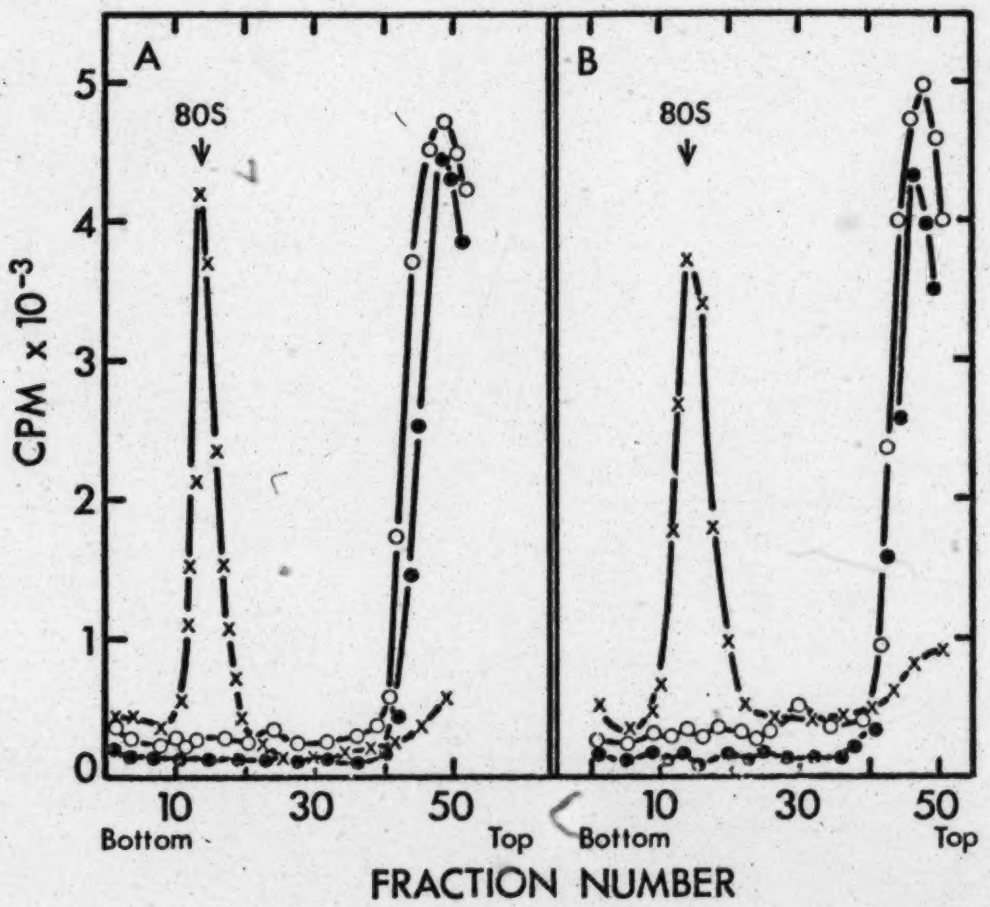


FIGURE 29: Sucrose density gradient centrifugation of ribosomes:[^{3}H]-mRNA: M-factor complex (Chapter 2, Section III (xv)). The reaction mixture (0.5 ml) for binding reaction as specified in the text was loaded onto a linear gradient of sucrose (8% to 15%) in a SW41 rotor cell (12 ml capacity), and spun at 37,000 r.p.m. in a Spinco Model L3-50 for 180 min at 4°C. Six-drop fractions were collected on filter discs, dried, and examined for [^{3}H]-radioactivity as described in Chapter 2, Section III (iv). A: [^{3}H]-poly-A binding to ribosomes. The reaction mixture contained ribosomes and [^{3}H]-poly-A (●); ribosomes, [^{3}H]-poly-A and the M_A-factor (X); ribosomes, [^{3}H]-poly-A and the M_U-factor (O). B: [^{3}H]-poly-U binding to ribosomes. The reaction mixture contained ribosomes and [^{3}H]-poly-U (●); ribosomes, [^{3}H]-poly-U and the M_A-factor (O); ribosomes, [^{3}H]-poly-U and the M_U-factor (X).

of the poly-U binding factor (M_U) or poly-U in the presence of the poly-A binding factor (M_A). The only combinations of components which resulted in mRNA:ribosome complex formation were those which included ribosomes, [^{3}H]-mRNA, and the corresponding homologous mRNA binding factor; that is, poly-A:ribosome:M_A-factor, and poly-U: ribosome:M_U-factor. These results clearly suggest that the mRNA binding proteins, as detected by Millipore filtration during the isolation procedure, mediate mRNA binding to ribosomes as analyzed by the sucrose density gradient method. Moreover, the results also suggest that poly-A and poly-U binding factors are specific to polyadenylate and polyuridylate respectively.

(iv) Specificity of M_A- and M_U- Factors

The specificity of M_A- and M_U- factors toward other synthetic mRNA's was critically examined using the Millipore filtration method (Chapter 2, Section III (xv)). The use of the filtration method was justified by the fact that the sucrose density gradient analysis revealed the specific complex formation between poly-U:ribosome:M_U-factor, as expected from the results of filtration experiments for the specific binding of M_U-factor and poly-U. The same situation was true for the M_A-factor:poly-A system.

As summarized in Table 11, M_A-factor: [^{3}H]-poly-A complex formation was diminished only by adding non-radioactive poly-A to the reaction mixture (the chase phenomenon). The other three non-radioactive homo-

TABLE 11

Specificity of M_A and M_U towards Homopolynucleates

Experiment	^3H mRNA (~0.1 µg)	M-factor (10 µg)	Unlabelled* polynucleate	% CPM bound (100%=15,000 c.p.m.)
Ia	Poly-A	M_A	-	100%
b	Poly-A	M_A	Poly-A	7%
c	Poly-A	M_A	Poly-C	93%
d	Poly-A	M_A	Poly-G	103%
e	Poly-A	M_A	Poly-U	96%
IIa	Poly-U	M_U	-	100%
b	Poly-U	M_U	Poly-A	102%
c	Poly-U	M_U	Poly-C	97%
d	Poly-U	M_U	Poly-G	104%
e	Poly-U	M_U	Poly-U	13%

* Unlabelled polynucleate was added to the reaction mixture 5 minutes after initiation of the reaction. The reaction was allowed to proceed for an additional 5 minutes and then the mixture was filtered through Millipore filters as described in Chapter 2, Section III (xv).

polynucleates had no effect whatsoever. Similarly, M_U-factor: [^{3}H]-poly-U complex formation was diminished only by adding non-radioactive poly-U. Therefore, it was concluded that M_A- and M_U-factors possess a stringent specificity toward poly-A and poly-U, respectively.

It should be noted that if [^{3}H]-poly-A was mixed with poly-U and heated to 37°C, no binding reaction occurred. Similarly, a [^{3}H]-poly-U and poly-A mixture at 37°C prevented the binding reaction. However, keeping all reaction components at 4°C permitted specific mRNA factor binding as shown in Table 11. This phenomenon was interpreted as lack of mRNA binding due to the formation of double-stranded RNA.

(v) [^{3}H]-Aminoacyl-tRNA Binding to mRNA:Ribosome:M-Factor Complex

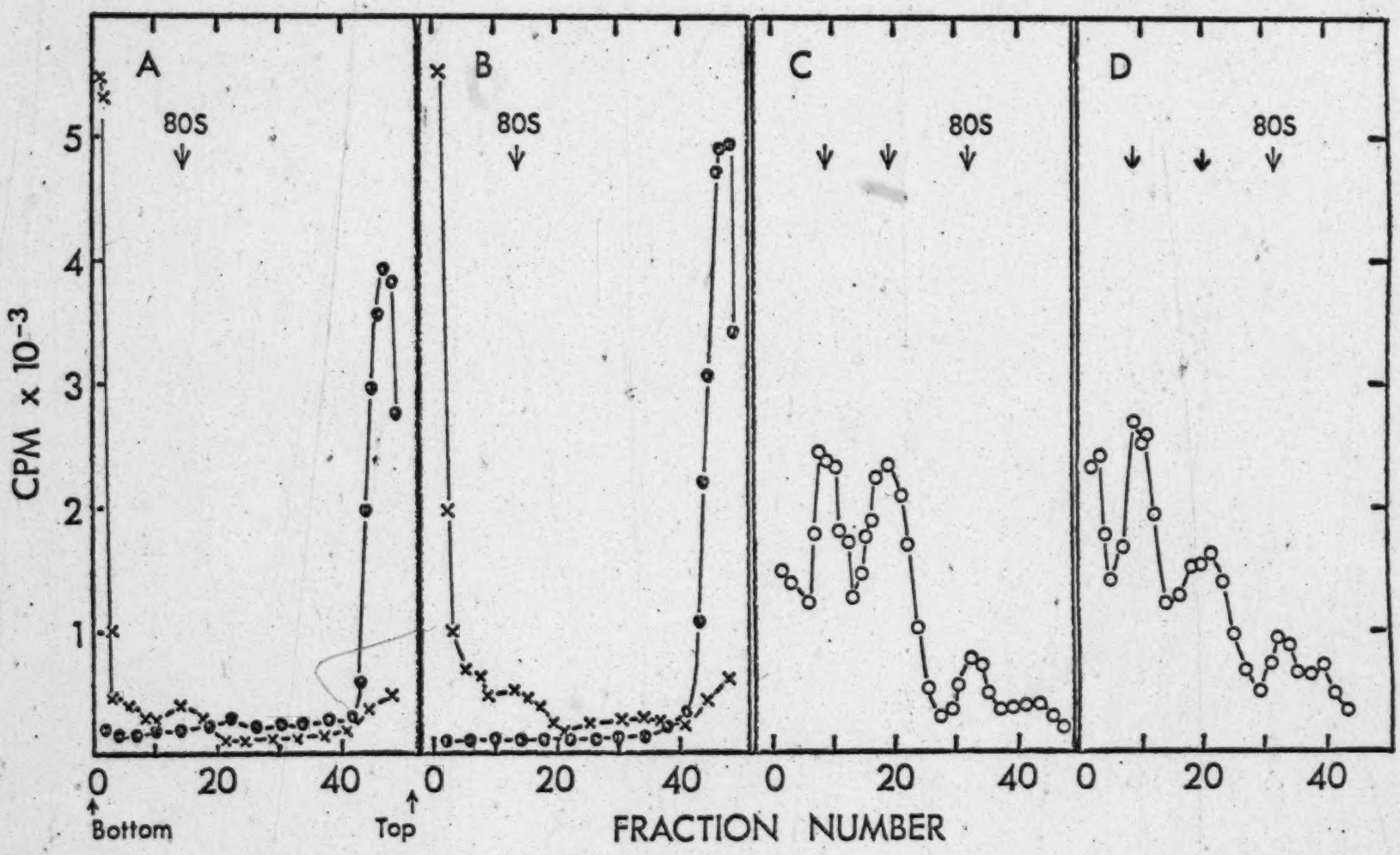
Demonstration of mRNA:ribosome complex formation in a cell-free system does not necessarily reflect a natural event taking place during the initial step of protein synthesis in the cell. The complex could represent an artifact of the cell-free system under study. As mentioned in Chapter 1, it was necessary to ensure that the factor mediated formation of mRNA:ribosome complex was a biologically significant event. It was sufficient to demonstrate that the ternary complex of M-factor: mRNA:ribosome could proceed to the next step of protein synthesis; that is, the specific binding of aminoacyl-tRNA as coded for by the mRNA employed in the system.

First, the binding of [^{3}H]-phenylalanyl-tRNA (25,000 c.p.m.) to the ribosomes in the presence of poly-U and M_U-factor was examined on sucrose density gradients as described in Chapter 2, Section III (xv).

No [^{3}H]-phenylalanyl-tRNA binding to the ribosomal complex was detected (Figure 30). Similarly no [^{3}H]-lysyl-tRNA binding was detected in the system containing [^{3}H]-lysyl-tRNA, ribosomes, poly-A, and M_A-factor (Figure 30). These results suggest two possibilities: (1) the mRNA binding to the ribosomes observed under the conditions described represents an artifact, or (2) the specific binding of aminoacyl-tRNA to the ribosome complex requires the presence of an additional factor(s).

The second possibility was considered first since a protein mixture from a 1 M KCl wash of crude ribosomes was found to contain aminoacyl-tRNA binding capacity by Millipore filtration. This fraction was purified and an aminoacyl-tRNA binding protein(s) was isolated as described in Chapter 2, Section III (ix). 20 µg of this aminoacyl-tRNA binding protein(s) was included in the specific binding reaction mixture containing ribosomes, M_U-factor, poly-U, and [^{3}H]-phenylalanyl-tRNA. The reaction mixture was incubated in the cold for 5 minutes and analyzed for specific binding by sucrose density gradient centrifugation for 180 minutes. The results (Figure 30) revealed that essentially 100% of the [^{3}H]-phenylalanyl-tRNA precipitated to the bottom of the gradient tube. This result suggested two possibilities: (1) aggregation of another component with [^{3}H]-phenylalanyl-tRNA, or (2) association of [^{3}H]-phenylalanyl-tRNA with polyribosomes. In order to determine which of the two possibilities was correct, the same reaction mixture described above was analyzed by sucrose gradient centrifugation for 60 minutes

FIGURE 30: Aminoacyl-tRNA binding to ribosome:M-factor:mRNA complex in the presence of the aminoacyl-tRNA binding factor (Chapter 2, Section III (xv)). The reaction mixture (0.5 ml) for aminoacyl-tRNA binding as specified in the text was loaded onto a linear gradient of sucrose, (8 to 15%), in a SW 41 rotor cell (12 ml), and spun at 37,000 r.p.m. at 4°C in a Spinco Model L3-50. At the end of spin, six-drop fractions were collected on filter discs, dried, and examined for [^{3}H]-radioactivity as described in Chapter 2, Section III (iv). A: [^{3}H]-lys-tRNA binding to ribosomes. The reaction mixture contained ribosomes the M_A-factor, poly-A, and [^{3}H]-lys-tRNA (20,000 cpm) (●); ribosomes, the M_A-factor, poly-A, [^{3}H]-lys-tRNA and the aminoacyl-tRNA binding factor (X). Samples were spun for 180 min. B: [^{3}H]-Phe-tRNA binding to ribosomes. The reaction mixture contained ribosomes, the M_U-factor, poly-U and [^{3}H]-phe-tRNA (25,000 cpm) (●); ribosomes, the M_U-factor, poly-U, [^{3}H]-phe-tRNA and the aminoacyl-tRNA binding factor (X). Samples were spun for 180 min. C: [^{3}H]-lys-tRNA binding in the complete system (X of A) spun for 60 min. D: [^{3}H]-phe-tRNA binding in the complete system (X of B) spun for 60 min.


A
B
C
D
80S
80S
80S
80S
CPM x 10⁻³
5
4
3
2
1
0
20
40
Bottom
Top
FRACTION NUMBER

(Figure 30). The results obtained from this experiment demonstrated the presence of trimeric, dimeric, and monomeric ribosomes. All of these forms bound to [^{3}H]-phenylalanyl-tRNA in the presence of the M_U-factor, aminoacyl-tRNA binding protein(s) and poly-U. A reaction mixture containing poly-A, ribosomes, M_A-factor, aminoacyl-tRNA binding protein(s), and [^{3}H]-lysyl-tRNA was analyzed in the same manner (Figure 30). Binding of [^{3}H]-lysyl-tRNA to all three ribosome species - trimeric, dimeric, and monomeric-was observed. Therefore, from these results it is evident that M_A- and M_U-factors can function as mediators of mRNA binding but not of aminoacyl-tRNA binding, and that binding of aminoacyl-tRNA to the mRNA:ribosome complex requires an additional protein factor(s).

One question was still unanswered concerning the mRNA and the aminoacyl-tRNA binding experiments: why were polyribosomes observed in the reaction of [^{3}H]-aminoacyl-tRNA binding while only monosomes were detected in the [^{3}H]-mRNA binding experiments? Since by definition the presence of polysomes requires the existence of mRNA chains sufficiently long to accommodate more than one ribosome, the obvious approach to the question was to examine the size of the mRNA's used in the two types of experiments. This was done by examination of the sedimentation boundary profile of the mRNA's by analytical ultracentrifugation as described in Chapter 2, Section III (xvii). The conditions employed were identical to those described for rabbit liver tRNA. It was found that the non-radioactive poly-U and poly-A used in the aminoacyl-tRNA binding experiments were considerably larger in size than the corresponding [^{3}H]-mRNA's. The difference in chain length of the mRNA species may

account for the observed phenomena in Figures 29 and 30. However, in light of recent knowledge concerning the function of initiation factors from both E. coli (226) and eukaryotes (227), more fundamental aspects should not be overlooked. For example, it is possible that the presence of mRNA binding factors alone do not cause dissociation of ribosomes but that in the presence of added aminoacyl-tRNA binding factor(s), dissociation of ribosomes with subsequent formation of polysomes, occurs. The possibility of such an event taking place was examined as follows: first, ribosomes were incubated with purified M-factor under binding conditions and then subjected to sucrose density gradient centrifugation analysis (Figure 31). The results indicated no detectable dissociation of ribosomes. Secondly, ribosomes were incubated with partially purified aminoacyl-tRNA binding protein(s) and analyzed on sucrose density gradients; again no dissociation of ribosomes was detected (Figure 31). The addition of both the M-factor and the aminoacyl-tRNA binding protein(s) to the ribosome system did not alter the separation profile regardless of the binding conditions employed. From this data it was concluded that M-factor and the aminoacyl-tRNA binding factor(s) alone or in combination, do not dissociate the purified ribosomes under the conditions employed for mRNA- or aminoacyl-tRNA binding.

Based on the above analysis the following conclusions can be drawn: (1) the purified rabbit liver ribosomes prepared according to the procedure specified in Chapter 5 are able to bind mRNA when the reaction

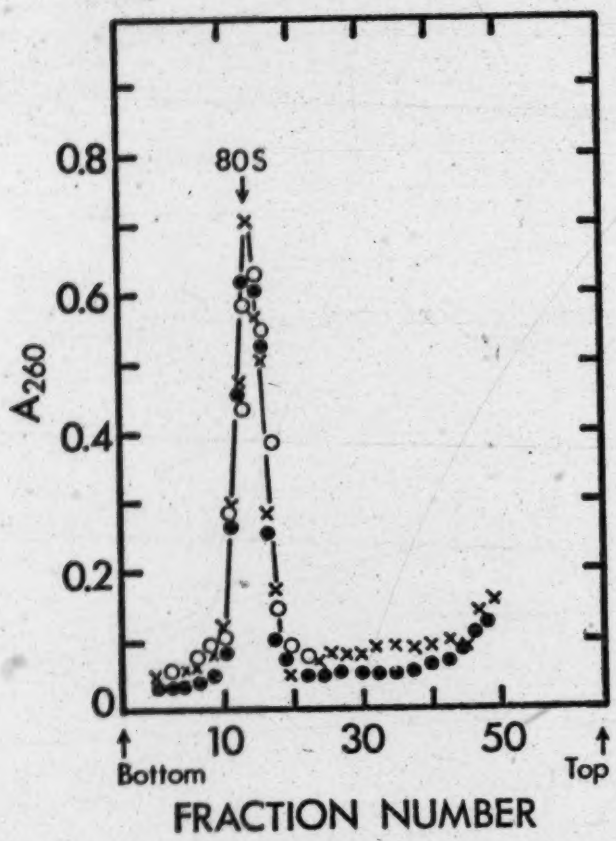


FIGURE 31: Inability of the M-factors and the aminoacyl-tRNA binding factor(s) to dissociate rabbit liver ribosomes. The reaction mixture (0.5 ml) for the binding study was incubated at 4°C for 5 min, loaded onto a sucrose gradient (8-15%), and spun for 180 min under the identical conditions described in Figs. 29 and 30. Six-drop fractions were collected, and absorption at 260 nm was examined after appropriate dilution with Buffer TR1. The reaction mixture contained 10 A_{260} units of ribosomes and 50 µg each of M_A- and M_U- factors (●); ribosomes and 50 µg of the aminoacyl-tRNA binding factor (O); ribosomes, the aminoacyl-tRNA binding factor and M-factors (X).

mixture is supplemented with the M-factor specific to that mRNA, and (2) the resulting ternary complex, mRNA:ribosome:M-factor, can subsequently bind aminoacyl-tRNA as specified by the genetic code when the reaction mixture is supplemented with an aminoacyl-tRNA binding protein(s). Both binding of mRNA and aminoacyl-tRNA occur in the cold (0-4°C).

(vi) Effect of Aurintricarboxylic Acid (ATA) on mRNA Binding

ATA is a potent inhibitor of the initiation of protein biosynthesis in the E. coli (182), wheat embryo (228) and mammalian systems (229). As mentioned in Chapter 4, the inhibitory effect of the dye was originally believed to be specific towards mRNA binding to ribosomes. However recent studies (Chapter 4) demonstrated that ATA is a non-specific inhibitor which interferes with a variety of reactions involving RNA-protein interactions. It is noteworthy that in all these studies the site of ATA binding was found to be on the enzyme. For this reason, the effect of ATA on mRNA:ribosome complex formation was re-examined. The main objective of the following studies was to determine whether ATA inhibited mRNA:M-factor complex formation or the binding of mRNA: M-factor complex to ribosomes.

First, the ATA effect on mRNA:M-factor complex formation was examined using the Millipore filtration method. As illustrated in Figure 32 both poly-U:M_U-factor and poly-A:M_A-factor complex formations were inhibited by ATA at approximately 10^{-6} M (50% inhibition value, designated as I_{50}). In these experiments 10 µg of the required M-factor in 0.5 ml of reaction mixture was used. This represents a molar

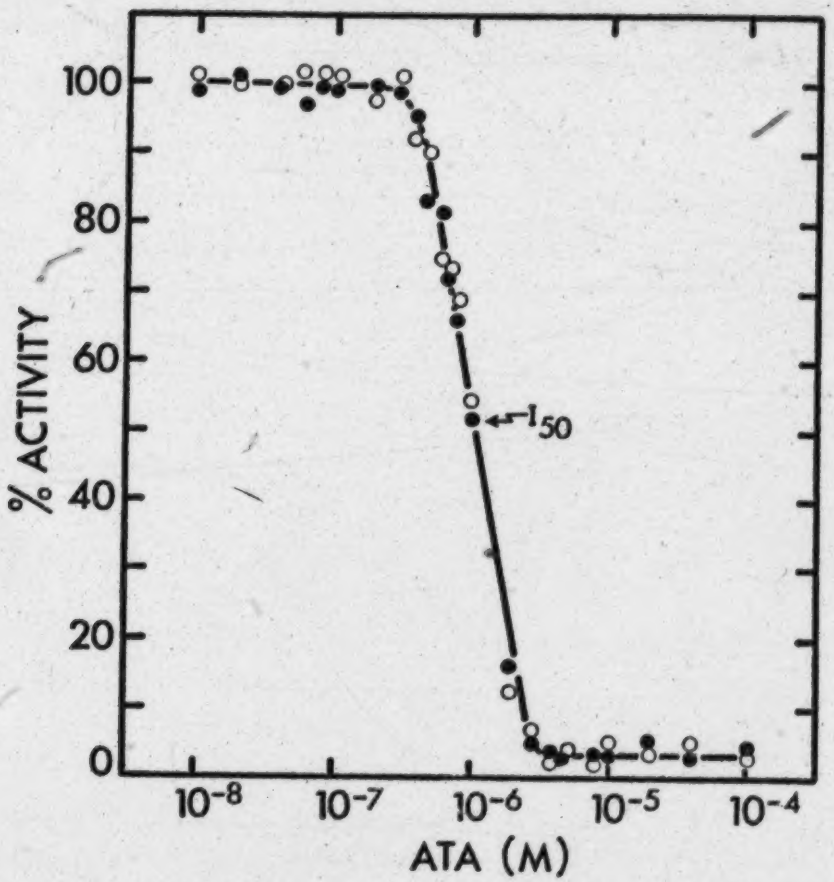


FIGURE 32: Inhibition of M-factor:mRNA complex formation by ATA_3. The binding of [^{3}H]-poly-A to the M_A-factor (●), and [^{3}H]-poly-U to the M_U-factor (0) as measured by the Millipore filtration method (Chapter 2, Section III (xv)). Each reaction mixture contained 10 μg of the M-factor, 30 mμCi of corresponding [^{3}H]-mRNA and varied concentrations of ATA as indicated on the abscissa.

ratio of ATA to M-factor, (estimated native molecular weight equals 180,000), of 10 in solution. This ratio is in close agreement with the phenylalanyl-tRNA synthetase system (Chapter 4, Section II (iv-c)). It should be pointed out that by increasing the amount of M-factor from 10 µg to 50 µg, ATA inhibition was reduced to 10%. However, increasing amounts of polynucleates did not alter the I_{50}. Therefore, these results suggest that there are multiple ATA binding sites on the M-factor itself.

In the next series of experiments, the order of adding reaction components was manipulated as described in Table 12 in order to obtain further information concerning the mode of ATA inhibition. ATA was found to exert its inhibitory action when the M-factor interacted first with ATA and then with mRNA. However, its inhibitory effect was reduced when the M-factor was allowed to interact first with mRNA and then with ATA. These facts indicated that the preformed complex was resistant to ATA action and that the site of interaction was on the M-factor.

Using the sucrose density gradient method, the effect of ATA on the formation of the ternary complex between mRNA:ribosomes:M-factor was examined. Figure 33 demonstrates that ATA (10^{-6} M) does not disrupt the preformed ternary complex. ATA inhibited the formation of the ternary complex when mixed with the M-factor prior to the addition of other components. However, ATA does not interfere with ternary complex formation when ribosomes are exposed to ATA prior to the addition of the mRNA:M-factor complex. These results provided further evidence

TABLE 12

Change in Efficiency of ATA Action by Modifying the Order of Adding Reaction Components

Experiment	Order of Addition 1st	2nd	3rd	[^{3}H]mRNA bound in CPM	% CPM
Ia	M_A-factor	[^{3}H]-Poly-A	-	30,169	100%
b	M_A-factor	[^{3}H]-Poly-A	ATA	22,319	73%
c	ATA	[^{3}H]-Poly-A	M_A-factor	13,576	45%
d	M_A-factor	ATA	[^{3}H]-Poly-A	2,689	9%
IIa	M_U-factor	[^{3}H]-Poly-U	-	34,049	100%
b	M_U-factor	[^{3}H]-Poly-U	ATA	22,979	68%
c	ATA	[^{3}H]-Poly-U	M_U-factor	14,641	43%
d	M_U-factor	ATA	[^{3}H]-Poly-U	2,379	7%

The addition of the three components in Ic and IIc is the regular order used for determination of I_{50} (Fig. 32).

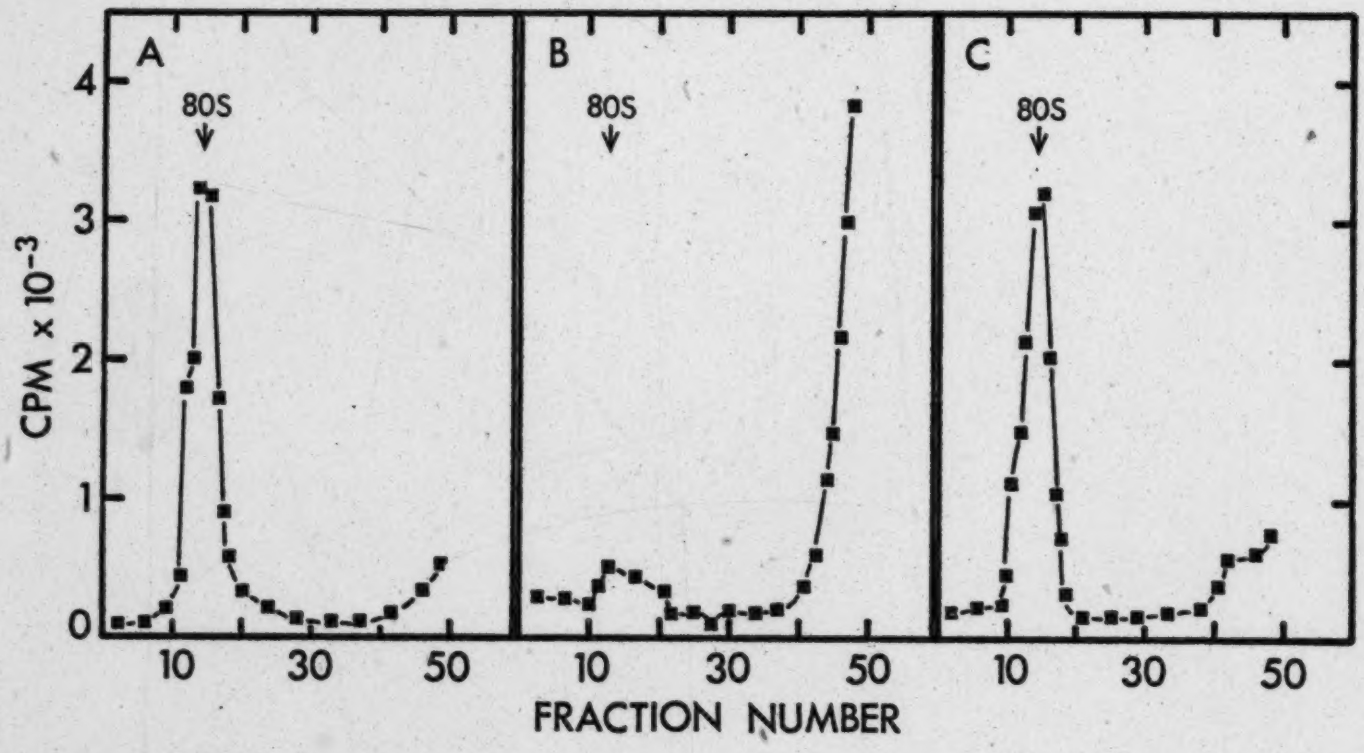


FIGURE 33: Sucrose density gradient analysis of ATA effect on the ternary complex formation between mRNA, ribosomes, and M-factor (Chapter 2, Section III (xv). The reaction mixture and centrifugation conditions were identical to those in Fig. 29, except that ATA (10^{-6}M) was added. (A) Ribosomes M_U-factor and [^{3}H]-poly-U were mixed and allowed to stand at 4°C for 5 min; then ATA was added. (B) M_U-factor and ATA were mixed and allowed to stand for 5 min; ribosomes and [^{3}H]-poly-U were separately incubated; then the two portions were mixed together and incubated for a further 5 min. (C) Ribosomes:ATA and M_U-factor:[^{3}H]-poly-U were individually pre-incubated and then mixed. Almost identical results were obtained in the [^{3}H]-poly-A:M_A-factor system (not shown).

that M-factor:mRNA interaction is sensitive to ATA. These results also led to an important conclusion: the interaction of the ribosome with the M-factor:mRNA complex is resistant to ATA action.

Although it was desirable to obtain information concerning the mode of ATA inhibition such as whether it is a competitive, non-competitive, or uncompetitive type, the binding reaction is not amenable to standard initial velocity studies and analysis by the Lineweaver-Burk plot.

III. Discussion

Cell-free studies using purified protein-synthesizing components have provided information as to step-by-step events at the stage of initiation of protein synthesis. There are at least three main factors involved in the initiation complex formation as revealed first in E. coli: IF-1, IF-2, and IF-3 (49-61). In eukaryotes, the counterparts have also been purified and are designated as IF-M_1, IF-M_2 and IF-M_3 (or IF-E, IF-E_2, and IF-E_3, depending on the laboratory (129-134,230). Of these factors, IF-3, which may be composed of several groups of polypeptides (231), and IF-M_3 are directly responsible for natural mRNA binding to permit formation of a ternary complex, mRNA:ribosome:initiator tRNA (62,135). Although additional factors, such as factor i, may modify cistron or messenger specificity (232), it is known that a given species of IF-3 (IF-M_3 in eukaryotes), permits the binding of a number of mRNA's (231-235) Therefore, cell-free studies have not proven that there are as many specific binding factors as the number of cistrons of a given organism.

Our original design of a cell-free protein synthesizing system did not incorporate any cistron-specific binding factors. However, we were forced to study the messenger-specific mRNA binding factors because the rabbit liver ribosomes isolated in our laboratory (127) do not bind with any synthetic mRNA's. Four kinds of mRNA binding factor were found in the post-ribosomal supernatant proteins, each of which is specific to one of: poly-A, poly-C, poly-G, and poly-U. They are designated as M_A-, M_C-, M_G-, and M_U-factors respectively. Of these four, M_A- and M_U- factors were purified to homogeneity as determined by SDS-gel electrophoresis and mRNA specificity studies. These studies resulted in the finding of base specific mRNA binding factors, speciec which had not been reported elsewhere.

During attempts to determine the molecular weight of the native M-factors by molecular sieving, an unusual property of the mRNA binding factors was recognized. As described in Section (i), after a series of DEAE-cellulose column chromatographies, each factor (M_A- and M_U-) was chromatographed on molecular sieiving columns. The molecular weight of the active form was estimated to be 180,000 by Sepharose 6B column co-chromatography with four marker proteins. Since globular proteins smaller than 200,000 can be fractionated on Sephadex G-200, the factor was re-chromatographed on this material. The result was that the factor was excluded from the column.

Therefore, the molecular weight of the active form of the factors could not be assessed at this stage. It is conceivable that these factors could possess unusual electronic charges which cause them to be excluded from the Sephadex G-200, or they could be non-globular proteins. Regardless of the problems in assessing the molecular weight of the active species of these factors, it is clear that the molecular weight of the polypeptides is 60,000, based on SDS-gel analysis. Although M_A- and M_U-factors possess the same polypeptide chain molecular weight, each factor possesses a different net negative charge according to the results of ion-exchange cellulose chromatography.

Evaluation of the biological function of these M-factors requires careful consideration. Although the approach towards the analysis of the initiation mechanism are numerous, they are based on two orientations: (a) a study of the mRNA concerned, using either synthetic polyribonucleate or natural mRNA, and (b) a technical approach to this complex mechanism of initiation, progressing either from the complex entity to the individual steps involved, or from the logically-anticipated individual steps to the complex entity of initiation.

It should be pointed out that the translation of synthetic mRNA occurs at high concentrations of Mg^{2+} in the absence of initiator met-$tRNA_f$ and two of three known initiation factors. On the other hand, the natural mRNA-decoding system can be initiated at low concentrations of Mg^{2+} only when the initiator tRNA and all three initiation factors are

present. The differen es in these two systems may bring into question the significance of the results described in this chapter because we used synthetic polyribonucleates as mRNA. However, the use of homopolyribonucleates enabled us to detect the presence of mRNA binding factors specific to different homopolynucleates. The application of this new finding to the natural mRNA system requires further investigation.

In this regard, it is worth emphasizing that these M-factors are unique insofar as the M_A-factor specifically mediates poly-A binding to ribosomes, just as the M_U-factor specifically mediates poly-U binding. Moreover, poly-AU binding to ribosomes was found to be maximal in the presence of both M_A- and M_U- factors. There is strong indication that the homopolynucleate binding mediated by a specific factor is an in-vitro duplication of a natural phenomenon. Unfortunately, the M_C- and M_G- factors have not been purified to homogeneity. The unavailability of these two latter factors prevented us from demonstrating the possibility of natural mRNA binding when all four complimentary M-factors are present.

As to the technical approaches, numerous groups have studied the initiation steps as a whole and evaluated the function of various protein factors in terms of the extent of stimulation of the overall initiation complex formation and initial peptide bond formation. With this approach, it is rather difficult to pinpoint a specific mechanism mediated by a particular factor. However, the study described in this thesis concerns a single step involved in one complex feature of

initiation: the binding of mRNA to the ribosomes. This approach tends to encounter a number of artifacts which have no significance in natural events taking place during the initiation of protein synthesis. For example, there are numerous cytoplasmic proteins which can form complexes with RNA but which do not catalyze ribosome binding. Therefore, we carefully examined the functional significance of M-factors in terms of two biological reactions: (a) synthetic mRNA binding to ribosomes which cannot bind with mRNA in the absence of an added factor, and (b) the specific binding of aminoacyl-tRNA to the ternary complex of mRNA:ribosome:M-factor. Thus the function of M-factors appears to be biologically significant.

The relation of the described M-factors to other known protein factors involved in the initiation step is obscure. Functionally, there are three initiation factors in mammalian systems analogous to those in the E. coli system - IF-1, IF-2, and IF-3. Of these, the first two mediate the binding of initiator met-tRNA to the mRNA ribosome complex. Therefore, there is no functional resemblance between them and the described M-factors. The most likely candidate for comparison is therefore IF-3, which apparently has dual functions: dissociation of ribosomal subunits and the subsequent binding of mRNA to the small ribosomal subunits. However, there are two basic differences between IF-3 and M-factors: (a) the molecular weight of IF-3 is approximately 25,000 (49), while that of M-factors is 60,000; and (b) M-factors do not have the function of dissociating ribosomes as IF-3 does. It is obvious from Table 2 (Chapter 1) that M-factors do not have the function

of either EF-1 or EF-2. Therefore, M-factors appear to be different from all known factors defined to date.

Although the M-factors do not resemble the known factors as described above, it is possible that M-factors reflect the protein moiety of cytoplasmic mRNP. The unique feature of the messenger RNA's isolated from eukaryotes is that they appear to exist as RNA-protein complexes at all times, and that they undergo three structural changes during the passage from the nucleoplasmic site for transcription to the cytoplasmic sites for translation. The protein moiety of mRNA: protein complex appears to be specific to each stage of its life cycle; the protein component of heterologous nuclear RNP is different from the counterpart of free cytoplasmic mRNP (236,237), which is also different from the protein moiety isolated from polyribosomes (238,239). Although the above scheme may be subjected to a considerable modification in future, the overall feature is not only a reflection of post-transcriptional control of mRNA function at stage of transporting mRNA from nuclei to cytoplasm, but also a suggestion of translational control by means of protein regulators. In particular, the protein moiety of polysomal mRNP may facilitate cistron specific or messenger specific translation in a given tissue. This possibility has been tested in a number of cross-examinations using a cell-free system of one tissue and mRNA from different origins, such as calf lens mRNA in reticulocyte system (240), hemoglobin mRNA (reticulocyte) in rat liver lysate (241), Q_β bacteriophage RNA in KB cell lysate (242), and globin mRNA in the plant system (243). These results were not always consistent in supporting

the existence of such a cistron-specific translation factor, although some results were taken as an absolute proof for the existence (244, 245). Unfortunately, the majority of these studies employed relatively crude cell-free systems, such as cell-lysates, so that the information obtained does not permit critical analysis of events taking place in the system or convincing correlation of M-factors with polysomal mRNA protein regulators.

The only remaining possibility concerning correlation of M-factors with other proteins is that M-factor may be equivalent to one of the protein subunits of E. coli ribosomes, S1, which is actually the site of mRNA binding. Due to the lack of available information on mammalian ribosomal proteins, this possibility cannot be assessed.

One additional piece of information obtained in the mRNA:ribosome complex formation studies described in this thesis is that aurintricarboxylic acid, a known inhibitor of the initiation reaction, exerts its inhibitory action on mRNA:M-factor binding, not on the binding of the preformed mRNA:M-factor entity to ribosomes. This information may provide some insight into the mechanism of RNA-protein interactions.

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